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PATENT APPLICATION

**PRODUCTION OF ATTENUATED, HUMAN-BOVINE CHIMERIC
RESPIRATORY SYNCYTIAL VIRUS VACCINES**

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PRODUCTION OF ATTENUATED, HUMAN-BOVINE CHIMERIC RESPIRATORY SYNCYTIAL VIRUS VACCINES

CROSS-REFERENCES TO RELATED APPLICATIONS

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This application claims the benefit of U.S. Provisional Patent Application
No. 60/143,132, filed by Buchholz et al. on July 9, 1999.

BACKGROUND OF THE INVENTION

10 Human respiratory syncytial virus (HRSV) is the leading viral agent of
serious pediatric respiratory tract disease worldwide (Collins, et al., Fields Virology 2:1313-
1352, 1996). RSV outranks all other microbial pathogens as a cause of pneumonia and
bronchiolitis in infants under one year of age. Virtually all children are infected by two
years of age, and reinfection occurs with appreciable frequency in older children and young
15 adults (Chanock et al., in Viral Infections of Humans, 3rd ed., A.S. Evans, ed., Plenum
Press, N.Y., 1989). RSV is responsible for more than one in five pediatric hospital
admissions due to respiratory tract disease, and in the United States alone causes nearly
100,000 hospitalizations and 4,500 deaths yearly. (Heilman, J Infect Dis 161:402-6, 1990).
In addition, there is evidence that serious respiratory tract infection early in life can initiate
20 or exacerbate asthma (Sigurs, et al., Pediatrics 95:500-5, 1995).

While human RSV usually is thought of in the context of the pediatric
population, it also is recognized as an important agent of serious disease in the elderly
(Falsey, et al., J. Infect. Dis. 172:389-394, 1995). Human RSV also causes life-threatening
disease in certain immunocompromised individuals, such as bone marrow transplant
25 recipients (Fouillard, et al., Bone Marrow Transplant 9:97-100, 1992).

For treatment of human RSV, one chemotherapeutic agent, ribavirin, is
available. However, its efficacy and use is controversial. There are also licensed products
for RSV intervention which are composed of pooled donor IgG (Groothuis, et al. N Engl J
Med 329:1524-30, 1993) or a humanized RSV-specific monoclonal antibody. These are
30 administered as passive immunoprophylaxis agents to high risk individuals. While these
products are useful, their high cost and other factors, such as lack of long term effectiveness,
make them inappropriate for widespread use. Other disadvantages include the possibility of

transmitting blood-borne viruses and the difficulty and expense in preparation and storage. Moreover, the history of the control of infectious diseases, and especially diseases of viral origin, indicates the primary importance of vaccines.

5 Despite decades of investigation to develop effective vaccine agents against RSV, no safe and effective vaccine has yet been achieved to prevent the severe morbidity and significant mortality associated with RSV infection. Failure to develop successful vaccines relates in part to the fact that small infants have diminished serum and secretory antibody responses to RSV antigens. Thus, these individuals suffer more severe infections from RSV, whereas cumulative immunity appears to protect older children and adults
10 against more serious impacts of the virus.

The mechanisms of immunity in RSV infection have recently come into focus. Secretory antibodies appear to be most important in protecting the upper respiratory tract, whereas high levels of serum antibodies are thought to have a major role in resistance to RSV infection in the lower respiratory tract. RSV-specific cytotoxic T cells, another
15 effector arm of induced immunity, are also important in resolving an RSV infection. However, while this latter effector can be augmented by prior immunization to yield increased resistance to virus challenge, the effect is short-lived. The F and G surface glycoproteins are the two major protective antigens of RSV, and are the only two RSV proteins which have been shown to induce RSV neutralizing antibodies and long term
20 resistance to challenge (Collins et al., Fields Virology, Fields et al. eds., 2:1313-1352, Lippincott-Raven, Philadelphia, 1996; Connors et al., J. Virol. 65(3):1634-7, 1991). The third RSV surface protein, SH, did not induce RSV-neutralizing antibodies or significant resistance to RSV challenge.

An obstacle to developing live RSV vaccines is the difficulty in achieving an
25 appropriate balance between attenuation and immunogenicity, partly due to the genetic instability of some attenuated viruses, the relatively poor growth of RSV in cell culture, and the instability of the virus particle. In addition the immunity which is induced by natural infection is not fully protective against subsequent infection. A number of factors probably contribute to this, including the relative inefficiency of the immune system in restricting
30 virus infection on the luminal surface of the respiratory tract, the short-lived nature of local mucosal immunity, rapid and extensive virus replication, reduced immune responses in the young due to immunological immaturity, immunosuppression by transplacentally derived

maternal serum antibodies, and certain features of the virus such as a high degree of glycosylation of the G protein. Also, as will be described below, human RSV exists as two antigenic subgroups A and B, and immunity against one subgroup is of reduced effectiveness against the other.

5 Although RSV can reinfect multiple times during life, reinfections usually are reduced in severity due to protective immunity induced by prior infection, and thus immunoprophylaxis is feasible. A live-attenuated RSV vaccine would be administered intranasally to initiate a mild immunizing infection. This has the advantage of simplicity and safety compared to a parenteral route. It also provides direct stimulation of local respiratory
10 tract immunity, which plays a major role in resistance to RSV. It also abrogates the immunosuppressive effects of RSV-specific maternally-derived serum antibodies, which typically are found in the very young. Also, while the parenteral administration of RSV antigens can sometimes be associated with immunopathologic complications (Murphy et al., Vaccine 8(5):497-502, 1990), this has never been observed with a live virus.

15 A formalin-inactivated virus vaccine was tested against RSV in the mid-1960s, but failed to protect against RSV infection or disease, and in fact exacerbated symptoms during subsequent infection by the virus. (Kim et al., Am. J. Epidemiol., 89:422-434, 1969; Chin et al., Am J. Epidemiol., 89:449-463, 1969; Kapikian et al., Am. J. Epidemiol., 89:405-421, 1969).

20 More recently, vaccine development for RSV has focused on attenuated RSV mutants. Friedewald et al., (J. Amer. Med. Assoc. 204:690-694, 1968) reported a cold passaged mutant of RSV (cpRSV) which appeared to be sufficiently attenuated to be a candidate vaccine. This mutant exhibited a slight increased efficiency of growth at 26°C compared to its wild-type (wt) parental virus, but its replication was neither temperature
25 sensitive nor significantly cold-adapted. The cold-passaged mutant, however, was attenuated for adults. Although satisfactorily attenuated and immunogenic for infants and children who had been previously infected with RSV (i.e., seropositive individuals), the cpRSV mutant retained a low level virulence for the upper respiratory tract of seronegative infants.

30 Similarly, Gharpure et al., (J. Virol. 3:414-421, 1969) reported the isolation of temperature sensitive RSV (tsRSV) mutants which also were promising vaccine candidates.

upper respiratory tract (Collins et al., Vaccine 8:164-168, 1990) and inconsistent protection in the lower respiratory tract (Crowe et al., Vaccine 11:1395-1404, 1993).

Despite these various efforts to develop an effective RSV vaccine, no licensed vaccine has yet been approved for RSV. The unfulfilled promises of prior approaches underscores a need for new strategies to develop RSV vaccines, and in particular methods for manipulating recombinant RSV to incorporate genetic changes that yield new phenotypic properties in viable, attenuated RSV recombinants. However, manipulation of the genomic RNA of RSV and other non-segmented negative-sense RNA viruses has heretofore proven difficult. Major obstacles in this regard include non-infectivity of naked genomic RNA of these viruses, poor viral growth in tissue culture, lengthy replication cycles, virion instability, a complex genome, and a refractory organization of gene products.

Recombinant DNA technology has made it possible to recover infectious non-segmented negative-stranded RNA viruses from cDNA, to genetically manipulate viral clones to construct novel vaccine candidates, and to rapidly evaluate their level of attenuation and phenotypic stability (for reviews, see Conzelmann, J. Gen. Virol. 77:381-89, 1996; Palese et al., Proc. Natl. Acad. Sci. U.S.A. 93:11354-58, 1996). In this context, recombinant rescue has been reported for infectious respiratory syncytial virus (RSV), parainfluenza virus (PIV), rabies virus (RaV), vesicular stomatitis virus (VSV), measles virus (MeV), rinderpest virus and Sendai virus (SeV) from cDNA-encoded antigenomic RNA in the presence of essential viral proteins (see, e.g., Garcin et al., EMBO J. 14:6087-6094, 1995; Lawson et al., Proc. Natl. Acad. Sci. U.S.A. 92:4477-81, 1995; Radecke et al., EMBO J. 14:5773-5784, 1995; Schnell et al., EMBO J. 13:4195-203, 1994; Whelan et al., Proc. Natl. Acad. Sci. U.S.A. 92:8388-92, 1995; Hoffman et al., J Virol. 71:4272-4277, 1997; Pecters et al., J. Virol. 73:5001-5009, 1999; Kato et al., Genes to Cells 1:569-579, 1996; Roberts et al., Virology 247(1), 1-6, 1998; Baron et al., J Virol. 71:1265-1271, 1997; International Publication No. WO 97/06270; U.S. Provisional Patent Application No. 60/007,083, filed September 27, 1995; U.S. Patent Application No. 08/720,132, filed September 27, 1996; U.S. Provisional Patent Application No. 60/021,773, filed July 15, 1996; U.S. Provisional Patent Application No. 60/046,141, filed May 9, 1997; U.S. Provisional Patent Application No. 60/047,634, filed May 23, 1997; U.S. Patent No. 5,993,824, issued November 30, 1999 (corresponding to International Publication No. WO 98/02530); U.S. Patent Application No. 09/291,894, filed by Collins et al. on April 13, 1999;

U.S. Provisional Patent Application No. 60/129,006, filed by Murphy et al. on April 13, 1999; Collins, et al., Proc Nat. Acad. Sci. USA 92:11563-11567, 1995; Bukreyev, et al., J Virol 70:6634-41, 1996, Juhasz et al., J. Virol. 71(8):5814-5819, 1997; Durbin et al., Virology 235:323-332, 1997; He et al. Virology 237:249-260, 1997; Baron et al. J. Virol. 71:1265-1271, 1997; Whitehead et al., Virology 247(2):232-9, 1998a; Buchholz et al. J. Virol. 73:251-9, 1999; Whitehead et al., J. Virol. 72(5):4467-4471, 1998b; Jin et al. Virology 251:206-214, 1998; and Whitehead et al., J. Virol. 73:(4)3438-3442, 1999, and Bukreyev, et al., Proc Nat Acad Sci USA 96:2367-72, 1999, each incorporated herein by reference in its entirety for all purposes).

Bovine RSV (BRSV), which is antigenically-related to human RSV (HRSV), offers an alternative approach to the development of a live attenuated virus vaccine for HRSV. The first vaccine used in humans, live vaccinia virus believed to be of bovine origin, was developed by Jenner almost 200 years ago for the control of smallpox. During the ensuing two centuries, vaccinia virus was successful in controlling this disease and played an essential role in the final eradication of smallpox. In this "Jennerian" approach to vaccine development, an antigenically-related animal virus is used as a vaccine for humans. Animal viruses that are well adapted to their natural host often do not replicate efficiently in humans and hence are attenuated. At present, there is a lack of a thorough understanding regarding the genetic basis for this form of host range restriction. Evolution of a virus in its animal or avian host results in significant divergence of nucleotide (nt) and amino acid sequences from that of the corresponding sequences in the related human virus. This divergent sequence, consisting of a large number of sequence differences, specifies the host range attenuation phenotype. Having an attenuation phenotype which is based on numerous sequence differences is a desirable property in a vaccine virus since it should contribute to the stability of the attenuation phenotype of the animal virus following its replication in humans.

The recently licensed quadrivalent rotavirus is an example of the Jennerian approach to vaccine development in which a nonhuman rotavirus strain, the rhesus rotavirus (RRV), was found to be attenuated in humans and protective against human serotype 3 to which it is antigenically highly related (Kapikian et al., Adv. Exp. Med. Biol. 327:59-69, 1992, incorporated herein by reference). Since there was a need for a multivalent vaccine that would induce resistance to each of the four major human rotavirus serotypes, the Jennerian approach was modified by constructing three reassortant viruses using

conventional genetic techniques of gene reassortment in tissue culture. Each single gene reassortant virus contained 10 RRV genes plus a single human rotavirus gene that coded for the major neutralization antigen (VP7) of serotype 1, 2, or 4. The intent was to prepare single gene substitution RRV reassortants with the attenuation characteristics of this simian virus and the neutralization specificity of human rotavirus serotype 1, 2, or 4. The quadrivalent vaccine based on the host range restriction of the simian RRV in humans provided a high level of efficacy against human rotavirus infection in infants and young children (Perez-Schael et al., N. Engl. J. Med. 337:1181-7, 1997, incorporated herein by reference).

However, the licensed vaccine retains mild reactogenicity in older seronegative infants lacking maternal antibody, therefore a second generation Jennerian vaccine, based on the UK strain of bovine rotavirus, is being developed to replace the RRV vaccine. The Jennerian approach also is being explored to develop vaccines for parainfluenza type 1 virus and for hepatitis A virus (Emerson et al., J. Infect. Dis. 173:592-7, 1996; Hurwitz et al., Vaccine 15, 533-40, 1997, each incorporated herein by reference). The Jennerian approach was used for the development of a live attenuated vaccine for influenza A virus but it failed to produce a consistently attenuated vaccine for use in humans (Steinhoff et al., Journal of Infectious Diseases 163:1023-1028, 1991, incorporated herein by reference).

Based on the foregoing developments, it is now possible to recover infectious RSV from cDNA and to design and implement various genetic manipulations to RSV clones to construct novel vaccine candidates. Thereafter, the level of attenuation and phenotypic stability, among other desired phenotypic characteristics, can be evaluated and adjusted. The challenge which thus remains is to develop a broad and diverse menu of genetic manipulations that can be employed, alone or in combination with other types of genetic manipulations, to construct infectious, attenuated RSV clones that are useful for broad vaccine use. In this context, an urgent need remains in the art for additional tools and methods that will allow engineering of safe and effective vaccines to alleviate the serious health problems attributable to RSV. Surprisingly, the present invention fulfills this need by providing additional tools for constructing infectious, attenuated RSV vaccine candidates.

Chimeric human-bovine RSV of the invention include a partial or complete “background” RSV genome or antigenome derived from or patterned after a human or bovine RSV strain or subgroup virus combined with one or more heterologous gene(s) or genome segment(s) of a different RSV strain or subgroup virus to form the human-bovine chimeric RSV genome or antigenome. In certain aspects of the invention, chimeric RSV incorporate a partial or complete bovine RSV background genome or antigenome combined with one or more heterologous gene(s) or genome segment(s) from a human RSV. In alternate aspects of the invention chimeric RSV incorporate a partial or complete human RSV background genome or antigenome combined with one or more heterologous gene(s) or genome segment(s) from a bovine RSV.

In exemplary embodiments, the invention is directed to infectious chimeric respiratory syncytial viruses (RSVs) that comprise a major nucleocapsid (N) protein, a nucleocapsid phosphoprotein (P), a large polymerase protein (L), a RNA polymerase elongation factor, and a partial or complete RSV background genome or antigenome of a human or bovine RSV combined with one or more heterologous gene(s) and/or genome segment(s) of a different RSV to form a human-bovine chimeric RSV genome or antigenome. The heterologous gene(s) and/or genome segment(s) that are useful within the invention include one or more RSV NS1, NS2, N, P, M, SH, M2(ORF1), M2(ORF2), L, F or G gene(s) or genome segment(s). Alternatively, heterologous genes and genome segments for incorporation within human-bovine chimeric RSV may include a leader, trailer or intergenic region of the RSV genome, or a segment thereof.

Within more detailed embodiments, human-bovine chimeric RSV of the invention incorporate one or more heterologous genes and/or genome segments that encode a RSV F, G and/or SH glycoprotein or an immunogenic domain or epitope thereof. Alternatively, the human-bovine chimeric RSV may incorporate a chimeric glycoprotein having both human and bovine glycoprotein domains or immunogenic epitopes. For example, the latter type of chimera may be constructed by incorporation into a bovine background genome or antigenome a heterologous genome segment encoding a glycoprotein ectodomain in proper reading frame with a genome segment encoding a functional remaining portion of the corresponding glycoprotein in the bovine genome or antigenome, whereby the resultant chimeric virus expresses a functional chimeric glycoprotein.

In other alternative embodiments of the invention, human-bovine chimeric RSV are provided wherein a human RSV is attenuated by incorporation of a selected bovine gene, genome segment, or plurality of genes or genome segments. In certain embodiments selected heterologous gene sets from BRSV are coordinately transferred into a HRSV background genome or antigenome. Exemplary bovine RSV genes from which individual or coordinately transferred groups of genes may be selected include the RSV N, P, NS1, NS2, M2-1 and M genes, which may be replaced singly or in any combination in a human RSV background genome or antigenome by one or more heterologous gene(s) from a bovine RSV to yield an attenuated chimeric derivative. In more detailed aspects, both N and P genes of a human RSV are replaced coordinately by counterpart N and P genes from a bovine RSV. This coordinate gene replacement is facilitated by functional cooperativity between certain genes in the RSV genome, which often arises in the case of neighboring gene pairs in the genome. Thus, in other alternative embodiments, both NS1 and NS2 genes of a human RSV are replaced by counterpart NS1 and NS2 genes from a bovine RSV. In yet additional embodiments, two or more of the M2-1, M2-2 and L genes of a HRSV are replaced by counterpart genes from a bovine RSV. For certain vaccine candidates within the invention for which a high level of host-range restriction is desired, each of the N, P, NS1, NS2, M2-1 and M genes of a human RSV are replaced by counterpart N, P, NS1, NS2, M2-1 and M genes from a bovine RSV.

Within a different aspect of the invention, human-bovine chimeric RSV are constructed wherein the chimeric genome or antigenome comprises a partial or complete bovine RSV background genome or antigenome combined with one or more heterologous gene(s) and/or genome segment(s) from a human RSV. In certain embodiments, one or more human RSV glycoprotein genes selected from F, G and SH, or one or more genome segment(s) encoding cytoplasmic domain, transmembrane domain, ectodomain or immunogenic epitope portion(s) of F, G, and/or SH is/are added or substituted within a partial or complete bovine RSV background genome or antigenome. For example, one or both human RSV glycoprotein genes F and G may be substituted to replace one or both counterpart F and G glycoprotein genes in a partial bovine RSV background genome or antigenome. Within these and related embodiments, the human-bovine chimeric genome or antigenome can incorporate antigenic determinants from one or both subgroup A and subgroup B human RSV. In more detailed aspects, both human RSV glycoprotein genes F and G are substituted to replace counterpart F and G glycoprotein genes in the bovine RSV

background genome or antigenome. An exemplary human-bovine chimeric RSV bearing these features describe in the examples below is rBRSV/A2.

Yet additional human-bovine chimeric RSV of the invention incorporate one or more human RSV glycoprotein genes selected from F, G and SH which are added or substituted at a position that is more promoter-proximal compared to a wild-type gene order position of a counterpart gene or genome segment within a partial or complete bovine RSV background genome or antigenome. In one such embodiment, both human RSV glycoprotein genes G and F are substituted at gene order positions 1 and 2, respectively, to replace counterpart G and F glycoprotein genes deleted at wild type positions 7 and 8, respectively in a partial bovine RSV background genome or antigenome. An exemplary human-bovine chimeric RSV bearing these features describe in the examples is rBRSV/A2-G1F2.

Coordinate gene transfers within human-bovine chimeric RSV are also directed to introduction of human antigenic genes within a bovine background genome or antigenome. In certain embodiments, one or more human RSV envelope-associated genes selected from F, G, SH, and M is/are added or substituted within a partial or complete bovine RSV background genome or antigenome. For example, one or more human RSV envelope-associated genes selected from F, G, SH, and M may be added or substituted within a partial bovine RSV background genome or antigenome in which one or more envelope-associated genes selected from F, G, SH, and M is/are deleted. In more detailed aspects, one or more genes from a gene set defined as human RSV envelope-associated genes F, G, and M are added within a partial bovine RSV background genome or antigenome in which envelope-associated genes F, G, SH, and M are deleted. An exemplary human-bovine chimeric RSV bearing these features describe in the examples below is rBRSV/A2-MGF.

As used herein, "RSV gene" generally refers to a portion of the RSV genome encoding an mRNA and typically begins at the upstream end with the 10-nucleotide gene-start (GS) signal and ends at the downstream end with the 12 to 13-nucleotide gene-end (GE) signal. Ten such genes for use within the invention are known for RSV, namely NS1, NS2, N, P, M, SH, G, F, M2 and L. The term "gene" is also used herein to refer to a "translational open reading frame" (ORF). ORF is more specifically defined as a translational open reading frame encoding a significant RSV protein, of which 11 are currently recognized: NS1, NS2, N, P, M, SH, G, F, M2-1 (alternatively, M2(ORF1)), M2-2 (alternatively,

M2(ORF2)), and L. Thus, the term "gene" interchangeably refers to a genomic RNA sequence that encodes a subgenomic RNA, and to a ORF (the latter term applies particularly in a situation such as in the case of the RSV M2 gene, where a single mRNA contains two overlapping ORFs that encode distinct proteins). Collins et al., J. Gen. Virol. 71:3015-3020, 5 1990; Bermingham and Collins, Proc. Natl. Acad. Sci. USA 96:11259-11264, 1999; Ahmadian et al., EMBO J. 19:2681-2689, 2000; Jin et al., J. Virol. 74:74-82, 2000 (each incorporated herein by reference). When the term "gene" is used in the context of determining gene position relative to a promoter position, the term ordinarily refers strictly to an mRNA-encoding sequence bordered by transcription gene-start and gene-end signal 10 motifs (Collins et al., Proc. Natl. Acad. Sci. USA 83:4594-4598, 1986; Kuo et al., J. Virol. 70:6892-6901, 1996; each incorporated herein by reference).

By "genome segment" is meant any length of continuous nucleotides from the RSV genome, which may be part of an ORF, a gene, or an extragenic region, or a combination thereof.

15 The partial or complete background genome or antigenome typically acts as a recipient backbone or vector into which are imported heterologous genes or genome segments of the counterpart, human or bovine RSV. Heterologous genes or genome segments from the counterpart, human or bovine RSV represent "donor" genes or polynucleotides that are combined with, added to, or substituted within, the background 20 genome or antigenome to yield a chimeric RSV that exhibits novel phenotypic characteristics compared to one or both of the contributing RSVs. For example, addition or substitution of heterologous genes or genome segments within a selected recipient RSV strain may result in an increase or decrease in attenuation, growth changes, altered immunogenicity, or another desired phenotypic changes as compared with a corresponding 25 phenotype(s) of the unmodified recipient and/or donor.

Genes and genome segments that may be selected for use as heterologous inserts or additions within the invention include genes or genome segments encoding a NS1, NS2, N, P, M, SH, M2(ORF1), M2(ORF2), L, F or G protein or portion thereof. Regulatory regions, such as the extragenic leader or trailer regions, can also be considered. In preferred 30 embodiments of the invention, chimeric RSV incorporates one or more heterologous gene(s) that encode an RSV F, G or SH glycoprotein. Alternatively, the chimeric RSV may incorporate a genome segment encoding a cytoplasmic domain, transmembrane domain,

ectodomain or immunogenic epitope of a RSV F, G or SH glycoprotein. These immunogenic proteins, domains and epitopes are particularly useful within human-bovine chimeric RSV because they generate novel immune responses in an immunized host. In particular, the G and F proteins, and immunogenic domains and epitopes therein, provide major neutralization and protective antigens. In addition, genes and genome segments encoding non-RSV proteins, for example, an SH protein as found in mumps and SV5 viruses, may be incorporated within human-bovine PIV of the invention. Regulatory regions, such as the extragenic 3' leader or 5' trailer regions, and gene-start, gene-end, intergenic regions, or 3' or 5' non-coding regions, are also useful as heterologous substitutions or additions.

For example, addition or substitution of one or more immunogenic gene(s) or genome segment(s) from a human RSV subgroup or strain to or within a bovine recipient genome or antigenome yields a recombinant, chimeric virus or subviral particle capable of generating an immune response directed against the human donor virus, including one or more specific human RSV subgroups or strains, while the bovine backbone confers an attenuated phenotype making the chimera a useful candidate for vaccine development. In one such exemplary embodiment, one or more human RSV glycoprotein genes F, SH, and/or G are added to or substituted within a partial or complete bovine genome or antigenome to yield an attenuated, infectious human-bovine chimera that elicits an anti-human RSV immune response in a susceptible host. In alternate embodiments human-bovine chimeric RSV additionally incorporates a gene or genome segment encoding an immunogenic protein, protein domain or epitope from multiple human RSV strains, for example two F or G proteins or immunogenic portions thereof from both RSV subgroups A and B. In yet additional alternate embodiments a human-bovine chimeric RSV genome or antigenome encodes a chimeric glycoprotein in the recombinant virus or subviral particle having both human and bovine glycoprotein domains or immunogenic epitopes. For example, a heterologous genome segment encoding a glycoprotein ectodomain from a human RSV F, SH or G glycoprotein may be joined with a genome segment encoding corresponding bovine F, SH or G glycoprotein cytoplasmic and endodomains in the background bovine genome or antigenome.

According to the methods of the invention, human-bovine chimeric RSV may be constructed by substituting the heterologous gene or genome segment for a

counterpart gene or genome segment in a partial RSV background genome or antigenome. Alternatively, the heterologous gene or genome segment may be added as a supernumerary gene or genome segment in combination with a complete (or partial if another gene or genome segment is deleted) RSV background genome or antigenome. For example, two human RSV G or F genes or genome segments can be included, one each from RSV subgroups A and B.

Often, a heterologous gene or genome segment is added at an intergenic position within a partial or complete RSV background genome or antigenome.

Alternatively, the gene or genome segment can be placed in other noncoding regions of the genome, for example, within the 5' or 3' noncoding regions or in other positions where noncoding nucleotides occur within the partial or complete genome or antigenome. In one aspect, noncoding regulatory regions contain cis-acting signals required for efficient replication, transcription, and translation, and therefore represent target sites for modification of these functions by introducing a heterologous gene or genome segment or other mutation as disclosed herein. In more detailed aspects of the invention, attenuating mutations are introduced into cis-acting regulatory regions to yield, e.g., (1) a tissue specific attenuation (Gromeier et al., J. Virol. 73:958-64, 1999; Zimmermann et al., J. Virol. 71:4145-9, 1997), (2) increased sensitivity to interferon (Zimmermann et al., J. Virol. 71:4145-9, 1997), (3) temperature sensitivity (Whitehead et al., Virology 247:232-9, 1998), (4) a general restriction in level of replication (Men et al., J. Virol. 70:3930-7, 1996; Muster et al., Proc. Natl. Acad. Sci. USA 88:5177-5181, 1991), and/or (5) host specific restriction of replication (Cahour et al., Virology 207:68-76, 1995). These attenuating mutations can be achieved in various ways to produce an attenuated human-bovine chimeric RSV of the invention, for example by point mutations, exchanges of sequences between related viruses, or deletions.

In yet additional alternative methods provided herein, a heterologous gene or genome segment may be added or substituted at a position corresponding to a wild-type gene order position of a counterpart gene or genome segment within the partial or complete RSV background genome or antigenome. In other embodiments, the heterologous gene or genome segment is added or substituted at a position that is more promoter-proximal or promoter-distal compared to a wild-type gene order position of a counterpart gene or genome segment within the background genome or antigenome, to enhance or reduce expression, respectively, of the heterologous gene or genome segment.

In general aspects of the invention, bovine genes or genome segments may be added to or substituted within a human RSV background to form an attenuated, human-bovine chimeric RSV. Alternatively, the chimera may be comprised of one or more human gene(s) or genome segment(s) added to or substituted within a bovine RSV background to form an attenuated RSV vaccine candidate. In this context, a chimeric RSV genome or antigenome is formed of a partial or complete bovine RSV background genome or antigenome combined with a heterologous gene or genome segment from a human RSV. The chimeric RSV thus formed may incorporate a heterologous gene or genome segment from a subgroup A or subgroup B human RSV, or from a specific human RSV strain. In preferred aspects, one or more human RSV glycoprotein genes F, G and, SH or a genome segment encoding a cytoplasmic domain, transmembrane domain, ectodomain or immunogenic epitope of a human RSV glycoprotein gene is substituted for a counterpart gene or genome segment within the bovine RSV background genome or antigenome.

In exemplary embodiments one or both of the human RSV glycoprotein genes F and G is/are substituted to replace one or both counterpart F and G glycoprotein genes in a bovine RSV background genome or antigenome. In a specific example described below, both human RSV glycoprotein genes F and G are substituted to replace counterpart F and G glycoprotein genes in the bovine RSV background genome or antigenome. In a parallel fashion, the chimeric human-bovine RSV of the invention can be readily designed to incorporate antigenic determinants from more than one RSV strain or subgroup, e.g., both human RSV subgroup A and subgroup B.

In additional aspects of the invention, attenuated, human-bovine chimeric RSV are produced in which the chimeric genome or antigenome is further modified by introducing one or more attenuating mutations specifying an attenuating phenotype in the resultant virus or subviral particle. These attenuating mutations may be generated de novo and tested for attenuating effects according to a rational design mutagenesis strategy. Alternatively, the attenuating mutations may be identified in existing biologically derived mutant RSV and thereafter incorporated into a human-bovine chimeric RSV of the invention.

The invention provides a new basis for the attenuation of a live virus vaccine against RSV and other pathogens, which attenuation is based in part on host range effects. Earlier studies involved swapping of an extragenic leader region of BRSV (45 nucleotides)

with a corresponding sequence from HRSV (44 nucleotides) (Buchholz et al, J. Virol. 73:251-259, 1999). Neither sequence encodes a protein, and no significant phenotypic effect was observed. The instant disclosure provides attenuated, chimeric RSV by the introduction of genome segments, entire genes or multiple genes between HRSV and BRSV.

5 Host range differences between HRSV and BRSV are exemplified by the highly permissive growth of HRSV in chimpanzees compared to the barely detectable or undetectable growth of BRSV in the same animal. The chimpanzee is a widely accepted and faithful model of RSV infection and immunogenic activity in humans, exhibiting virus replication and disease comparable to that of humans. As illustrated herein below, host
10 range differences of chimeric RSV observed in chimpanzees are correlated with host range differences observed in cell culture, providing a convenient preliminary assay.

Host range effects observed in the chimeric, human-bovine RSV of the invention are generally related to nucleotide and amino acid sequence differences observed between HRSV and BRSV. For example, the percent amino acid identity between HRSV
15 and BRSV for each of the following proteins is: NS1 (69%), NS2 (84%), N (93%), P (81%), M (89%), SH (38%), G (30%), F (81%), M2-1 (80%), L (77%). Because of the extensive genetic divergence between HRSV and BRSV (replacement of the N gene of HRSV with that of BRSV, for example, involves approximately 26 amino acid differences), chimeric bovine-human RSV of the invention are particularly useful vaccine candidates. As
20 exemplified herein below, replacement of the BRSV G and F glycoproteins with those of HRSV increases the permissivity of recombinant BRSV for replication in chimpanzees. The involvement of multiple genes and genome segments each conferring multiple amino acid or nucleotide differences provides a broad basis for attenuation which is highly stable to reversion. This mode of attenuation contrasts sharply to HRSV viruses attenuated by one or
25 several point mutations, where reversion of an individual mutation will yield a significant or complete reacquisition of virulence. In addition, known attenuating point mutations in HRSV typically yield a temperature sensitive phenotype. This is because the temperature sensitive phenotype was specifically used as the first screen to identify altered progeny following exposure of HRSV to mutagens. One problem with attenuation associated with
30 temperature sensitivity is that the virus can be overly restricted for replication in the lower respiratory tract while being under attenuated in the upper respiratory tract. This is because there is a temperature gradient within the respiratory tract, with temperature being higher

conservative mutation), as described in U.S. Provisional Patent Application No. 60/129,006, filed by Murphy et al. on April 13, 1999, incorporated herein by reference.

Human-bovine chimeric RSV designed and selected for vaccine use often have at least two and sometimes three or more attenuating mutations to achieve a satisfactory level of attenuation for broad clinical use. In one embodiment, at least one attenuating mutation occurs in the RSV polymerase gene L (either in the donor or recipient gene) and involves one or more nucleotide substitution(s) specifying an amino acid change in the polymerase protein specifying an attenuation phenotype which may or may not involve a temperature-sensitive (ts) phenotype. Chimeric RSV of the invention may incorporate an attenuating mutation in any additional RSV gene besides L, e.g., in the M2 gene. However, preferred human-bovine chimeric RSV in this context incorporate one or more nucleotide substitutions in the large polymerase gene L resulting in an amino acid change at amino acid Asn43, Cys319, Phe 521, Gln831, Met1169, Tyr1321 and/or His 1690 in the RSV polymerase gene L, as exemplified by the changes, Ile for Asn43, Leu for Phe521, Leu for Gln831, Val for Met1169, and Asn for Tyr1321. Other alternative amino acid changes, particularly conservative changes with respect to identified mutant residues, at these positions can of course be made to yield a similar effect as the identified, mutant substitution. Additional desired mutations for incorporation into human-bovine chimeric RSV of the invention include attenuating mutations specifying an amino acid substitution at Val267 in the RSV N gene, Glu218 and/or Thr523 in the RSV F gene, and a nucleotide substitution in the gene-start sequence of gene M2. Any combination of one or more attenuating mutations identified herein, up to and including a full complement of these mutations, may be incorporated in human-bovine chimeric RSV to yield a suitably attenuated recombinant virus for use in selected populations or broad populations of vaccine recipients.

Attenuating mutations for incorporation in human-bovine chimeric RSV of the invention may be selected in coding portions of a donor or recipient RSV gene or in non-coding regions such as a cis-regulatory sequence. Exemplary non-coding mutations include single or multiple base changes in a gene start sequence, as exemplified by a single or multiple base substitution in the M2 gene start sequence at nucleotide 7605 (nucleotide 7606 in recombinant sequence).

5 Exemplary heterologous sequences include RSV sequences from one human RSV strain combined with sequences from a different human RSV strain in a human-bovine chimeric RSV. For example, chimeric RSV of the invention may incorporate sequences from two or more wild-type or mutant RSV strains, for example mutant strains selected from cpts RSV 248, cpts 248/404, cpts 248/955, cpts RSV 530, cpts 530/1009, or cpts 530/1030.

Alternatively, human-bovine chimeric RSV may incorporate sequences from two or more, wild-type or mutant human RSV subgroups, for example a combination of human RSV subgroup A and subgroup B sequences. In yet additional aspects, one or more human RSV coding or non-coding polynucleotides are substituted with a counterpart sequence from a heterologous RSV or non-RSV virus, alone or in combination with one or more selected attenuating mutations, e.g., cp and/or ts mutations, to yield novel attenuated vaccine strains.

Mutations incorporated within chimeric cDNAs, vectors and viral particles of the invention can be introduced individually or in combination into a full-length human-bovine chimeric RSV cDNA and the phenotypes of rescued virus containing the introduced mutations can be readily determined. In exemplary embodiments, amino acid changes displayed by attenuated, biologically-derived viruses versus a wild-type RSV, for example changes exhibited by cpRSV or tsRSV, are incorporated in combination within a recombinant human-bovine chimeric RSV to yield a desired level of attenuation.

In yet additional aspects of the invention, chimeric human-bovine RSV can be readily designed as “vectors” to incorporate antigenic determinants from different pathogens, including more than one RSV strain or group (e.g., both human RSV A and RSV B subgroups), human parainfluenza virus (HPIV) including HPIV3, HPIV2 and HPIV1, measles virus and other pathogens (see, e.g., U.S. Provisional Patent Application Serial No. 60/170,195; U.S. Patent Application No. 09/458,813; and U.S. Patent Application No. 09/459,062, each incorporated herein by reference). Within various embodiments, the bovine-human chimeric genome or antigenome comprises a partial or complete RSV “vector genome or antigenome” combined with one or more heterologous genes or genome segments encoding one or more antigenic determinants of one or more heterologous

pathogens. The heterologous pathogen may be a heterologous RSV (i.e., a RSV of a different strain or subgroup), and the heterologous gene or genome segment may encode a RSV NS1, NS2, N, P, M, SH, M2(ORF1), M2(ORF2), L, F or G protein or fragment (e.g., an immunogenic domain or epitope) thereof. For example, the vector genome or antigenome may be a partial or complete RSV A genome or antigenome and the heterologous gene(s) or genome segment(s) may encode antigenic determinant(s) of a RSV B subgroup virus.

In alternative embodiments, the human-bovine chimeric RSV vector genome or antigenome is a partial or complete BRSV genome or antigenome and the heterologous gene(s) or genome segment(s) encoding the antigenic determinant(s) is/are of one or more HRSV(s). For example, the partial or complete BRSV genome or antigenome may incorporate one or more gene(s) or genome segment(s) encoding one or more HRSV glycoprotein genes selected from F, G and SH, or one or more genome segment(s) encoding cytoplasmic domain, transmembrane domain, ectodomain or immunogenic epitope portion(s) of F, G, and/or SH of HRSV.

In other alternate embodiments, human-bovine chimeric RSV designed as "vectors" for carrying heterologous antigenic determinants incorporate one or more antigenic determinants of a non-RSV pathogen, such as a human parainfluenza virus (HPIV). In one exemplary embodiment, one or more HPIV1, HPIV2, or HPIV3 gene(s) or genome segment(s) encoding one or more HN and/or F glycoprotein(s) or antigenic domain(s), fragment(s) or epitope(s) thereof is/are added to or incorporated within the partial or complete HRSV vector genome or antigenome. In more detailed embodiments, a transcription unit comprising an open reading frame (ORF) of an HPIV1, HPIV2, or HPIV3 HN or F gene is added to or incorporated within the chimeric HRSV vector genome or antigenome.

In yet additional alternate embodiments, the vector genome or antigenome comprises a partial or complete HRSV or BRSV genome or antigenome and the heterologous pathogen is selected from measles virus, subgroup A and subgroup B respiratory syncytial viruses, mumps virus, human papilloma viruses, type 1 and type 2 human immunodeficiency viruses, herpes simplex viruses, cytomegalovirus, rabies virus, Epstein Barr virus, filoviruses, bunyaviruses, flaviviruses, alphaviruses and influenza viruses. Based on this exemplary list of candidate pathogens, the selected heterologous antigenic determinant(s) may be selected from measles virus HA and F proteins, subgroup A

or subgroup B respiratory syncytial virus F, G, SH and M2 proteins, mumps virus HN and F proteins, human papilloma virus L1 protein, type 1 or type 2 human immunodeficiency virus gp160 protein, herpes simplex virus and cytomegalovirus gB, gC, gD, gE, gG, gH, gI, gJ, gK, gL, and gM proteins, rabies virus G protein, Epstein Barr Virus gp350 protein; filovirus G protein, bunyavirus G protein, Flavivirus E and NS1 proteins, and alphavirus E protein, and antigenic domains, fragments and epitopes thereof. In one embodiment, the heterologous pathogen is measles virus and the heterologous antigenic determinant(s) is/are selected from the measles virus HA and F proteins and antigenic domains, fragments and epitopes thereof. To achieve such a chimeric construct, a transcription unit comprising an open reading frame (ORF) of a measles virus HA gene may be added to or incorporated within a HRSV vector genome or antigenome.

The present invention thus provides human-bovine chimeric RSV clones, polynucleotide expression constructs (also referred to as vectors) and particles which can incorporate multiple, phenotype-specific mutations introduced in selected combinations into the chimeric genome or antigenome to produce an attenuated, infectious virus or subviral particle. This process coupled with routine phenotypic evaluation provides human-bovine chimeric RSV having such desired characteristics as attenuation, temperature sensitivity, altered immunogenicity, cold-adaptation, small plaque size, host range restriction, etc. Mutations thus identified are compiled into a "menu" and introduced in various combinations to calibrate a vaccine virus to a selected level of attenuation, immunogenicity and stability.

In yet additional aspects of the invention, human-bovine chimeric RSV, with or without attenuating mutations, are constructed to have a nucleotide modification to yield a desired phenotypic, structural, or functional change. Typically, the selected nucleotide modification will specify a phenotypic change, for example a change in growth characteristics, attenuation, temperature-sensitivity, cold-adaptation, plaque size, host range restriction, or immunogenicity. Structural changes in this context include introduction or ablation of restriction sites into RSV encoding cDNAs for ease of manipulation and identification.

In preferred embodiments, nucleotide changes within human-bovine chimeric RSV include modification of a viral gene by deletion of the gene or ablation of its expression. Target genes for mutation in this context include the attachment (G) protein,

fusion (F) protein, small hydrophobic (SH), RNA binding protein (N), phosphoprotein (P), the large polymerase protein (L), the transcription elongation factor (M2 ORF1), the RNA regulatory factor M2 ORF2, the matrix (M) protein, and two nonstructural proteins, NS1 and NS2. Each of these proteins can be selectively deleted, substituted or rearranged, in whole or in part, alone or in combination with other desired modifications, to achieve novel chimeric RSV recombinants.

In one aspect of the invention, an SH, NS1, NS2, G or M2-2 gene is modified in the human-bovine chimeric RSV. For example, each of these genes may be deleted or its expression ablated (e.g., by introduction of a stop codon) to alter the phenotype of the resultant human-bovine chimeric RSV clone to improve growth, attenuation, immunogenicity or other desired phenotypic characteristics. For example, deletion of the SH gene in the background or heterologous (i.e. recipient or donor, respectively) portion of the recombinant genome or antigenome will yield a chimeric RSV having novel phenotypic characteristics such as enhanced growth *in vitro* and/or attenuation *in vivo*. In a related aspect, an SH gene deletion, or deletion of another selected non-essential gene or genome segment such as a NS1, NS2, G or M2-2 gene deletion is constructed in a human-bovine chimeric RSV, alone or in combination with one or more different mutations specifying an attenuated phenotype, e.g., a point mutation adopted directly (or in modified form, e.g., by introducing multiple nucleotide changes in a codon specifying the mutation) from a biologically derived attenuated RSV mutant. For example, the SH, NS1, NS2, G or M2-2 gene may be deleted in combination with one or more cp and/or ts mutations adopted from cpts248/404, cpts530/1009, cpts530/1030 or another selected mutant RSV strain, to yield a chimeric RSV having increased yield of virus, enhanced attenuation, improved immunogenicity and genetic resistance to reversion from an attenuated phenotype due to the combined effects of the different mutations.

Alternative nucleotide modifications can include a deletion, insertion, addition or rearrangement of a cis-acting regulatory sequence for a selected gene in the human-bovine chimera. In one example, a cis-acting regulatory sequence of one RSV gene is changed to correspond to a heterologous regulatory sequence, which may be a counterpart cis-acting regulatory sequence of the same gene in a different RSV or a cis-acting regulatory sequence of a different RSV gene. For example, a gene end signal may be modified by conversion or substitution to a gene end signal of a different gene in the same RSV strain. In

other embodiments, the nucleotide modification may comprise an insertion, deletion, substitution, or rearrangement of a translational start site within the chimeric genome or antigenome, e.g., to ablate an alternative translational start site for a selected form of a protein. In one example, the translational start site for a secreted form of the RSV G protein is ablated to modify expression of this form of the G protein and thereby produce desired *in vivo* effects.

In addition, a variety of other genetic alterations can be produced in a human-bovine chimeric RSV genome or antigenome, alone or together with one or more attenuating mutations adopted from a biologically derived mutant RSV. For example, genes or genome segments from non-RSV sources may be inserted in whole or in part. Alternatively, the order of genes can be changed, gene overlap removed, or an RSV genome promoter replaced with its antigenome counterpart. Different or additional modifications in the chimeric genome or antigenome can be made to facilitate manipulations, such as the insertion of unique restriction sites in various intergenic regions (e.g., a unique *StuI* site between the G and F genes) or elsewhere. Nontranslated gene sequences can be removed to increase capacity for inserting foreign sequences. In yet additional aspects, polynucleotide molecules or vectors encoding the chimeric RSV genome or antigenome can be modified to encode non-RSV sequences, e.g., a cytokine, a T-helper epitope, a restriction site marker, or a protein of a microbial pathogen (e.g., virus, bacterium or fungus) capable of eliciting a protective immune response in an intended host. In one such embodiment, human-bovine chimeric RSV are constructed that incorporate a gene or genome segment from a parainfluenza virus (PIV), for example a PIV HN or F glycoprotein or an immunogenic domain or epitope thereof.

All of the foregoing modifications within the human-bovine chimeric RSV genome or antigenome, including nucleotide insertions, rearrangements, deletions or substitutions yielding point mutations, site-specific nucleotide changes, and changes involving entire genes or genome segments, may be made to either the heterologous donor gene or genome segment, or the recipient, background genome or antigenome. In each case, these alterations will preferably specify one or more phenotypic change(s) in the resulting recombinant RSV, such as a phenotypic change that results in attenuation, temperature-sensitivity, cold-adaptation, small plaque size, host range restriction, alteration in gene expression, or a change in an immunogenic epitope.

The above methods and compositions for producing human-bovine chimeric RSV yield infectious viral or subviral particles, or derivatives thereof. An infectious virus is comparable to the authentic RSV virus particle and is infectious as is. It can directly infect fresh cells. An infectious subviral particle typically is a subcomponent of the virus particle which can initiate an infection under appropriate conditions. For example, a nucleocapsid containing the genomic or antigenomic RNA and the N, P, L and M2(ORF1) proteins is an example of a subviral particle which can initiate an infection if introduced into the cytoplasm of cells. Subviral particles provided within the invention include viral particles which lack one or more protein(s), protein segment(s), or other viral component(s) not essential for infectivity.

In other embodiments the invention provides a cell or cell-free lysate containing an expression vector which comprises an isolated polynucleotide molecule encoding a human-bovine chimeric RSV genome or antigenome as described above, and an expression vector (the same or different vector) which comprises one or more isolated polynucleotide molecules encoding the N, P, L and RNA polymerase elongation factor proteins of RSV. One or more of these proteins also can be expressed from the genome or antigenome cDNA. Upon expression the genome or antigenome and N, P, L, and RNA polymerase elongation factor proteins combine to produce an infectious human-bovine chimeric RSV viral or subviral particle.

Attenuated human-bovine chimeric RSV of the invention is capable of eliciting a protective immune response in an infected human host, yet is sufficiently attenuated so as to not cause unacceptable symptoms of severe respiratory disease in the immunized host. The attenuated chimeric virus or subviral particle may be present in a cell culture supernatant, isolated from the culture, or partially or completely purified. The virus may also be lyophilized, and can be combined with a variety of other components for storage or delivery to a host, as desired.

The invention further provides novel vaccines comprising a physiologically acceptable carrier and/or adjuvant and an isolated attenuated human-bovine chimeric RSV. In one embodiment, the vaccine is comprised of a human-bovine chimeric RSV having at least one, and preferably two or more attenuating mutations or other nucleotide modifications as described above. The vaccine can be formulated in a dose of 10^3 to 10^6 PFU of attenuated virus. The vaccine may comprise attenuated chimeric virus that elicits an

BRIEF DESCRIPTION OF THE DRAWINGS

Figs. 1A and 1B depict replacement of the G and F genes of recombinant (r) bovine RSV (BRSV) strain ATue51908 with the G and F genes of the human RSV (HRSV) strain A2 to create the recombinant chimeric virus rBRSV/A2.

Fig. 1A is a diagram of the rBRSV genome, illustrating the replacement of the F and G genes with those of HRSV. The location of the open reading frames (ORFs) are shown as shaded (BRSV) or open (HRSV) rectangles. The G and F genes are shown as enlargements, in which gene end/polyadenylation signals are represented by bars, gene start signals are shown as filled triangles, and the positions of synthetic restriction sites serving as genetic markers or as boundaries of swapped restriction fragments are indicated by arrowheads with the position in the complete antigenome sequence shown underneath. The size of the fragments framed by the marker restriction sites is indicated in parentheses. The genome length of the recombinant viruses is indicated on the left. The roman numerals (I, II and III) refer to intergenic regions which are expanded in Fig. 1B.

Fig. 1B illustrates alignment of the SH/G (I), G/F (II), and F/M2 (III) intergenic regions of a biologically-derived BRSV strain ATue51908 (top line); the recombinant version, rBRSV ATue51908 (second line), a recombinant chimeric virus rBRSV/A2 (third line), and biologically derived HRSV strain A2 (bottom line). Figure 1B, Panel I: SH/G intergenic region: ATue51908 (top line) (SEQ ID NO. 1); rBRSV (second line) (SEQ ID NO. 2); rBRSV/A2 (third line) (SEQ ID NO. 3); HRSV strain A2 (bottom line) (SEQ ID NO. 4). Figure 1B, Panel II: G/F intergenic region: ATue 51908 (top line) (SEQ ID NO. 5); rBRSV (second line) (SEQ ID NO. 6); rBRSV/A2 (third line) (SEQ ID NO. 7); HRSV A2 (bottom line) (SEQ ID NO. 8). Figure 1B, Panel 3: F/M2 intergenic region: ATue51908 (top line) (SEQ ID NO. 9); rBRSV (second line) (SEQ ID NO. 10); rBRSV/A2 (third line) (SEQ ID NO. 11); HRSV A2 (SEQ ID NO. 12). The sequences are shown as DNA positive strand. Gaps are indicated by dots, gene start and gene end signals are shaded, and translation start codons are in bold. Hash marks indicate nucleotide identity. Restriction sites are indicated, with recognition sequences shaded. Orf is open reading frame. Numbering refers to the genomic positions (for BRSV, strain ATue51908: GenBank accession no. AF092942; for HRSV strain A2: GenBank accession no. M74568).

Figs. 2A-2C demonstrate marker restriction sites in the genomes of biologically derived BRSV (ATue51908); its recombinant version rBRSV, and the recombinant chimera rBRSV-A2. RT-PCR was performed on total RNA of BSR T7/5 cells infected with the indicated virus. The PCR products were subjected to restriction analysis and analyzed on a 2% (Figs. 2A, 2B) or 3% agarose gel (Fig. 2C). Nucleotide lengths are estimates based on calculated sizes. In all cases, a PCR product was not detected when the RT step was omitted. M, 1 kb DNA ladder (Life Technologies, Gaithersburg, Maryland). The size of some marker fragments is indicated on the right.

Fig. 2A shows an analysis of RT-PCR products corresponding to positions 3612 to 4886 (ATue51908 and rBRSV) or positions 3612 to 4885 (rBRSV/A2), encompassing the Sall site (position 4673) contained in the SH/G intergenic regions of the recombinant virus isolates. The PCR products corresponding to the predicted size of approximately 1,273-1274 bp were digested with Sall. This did not cleave the biologically-derived ATue51908, which lacked this introduced site, whereas for the recombinant viruses this resulted in bands of 1,061 bp (both viruses) and 213 bp (rBRSV) or 212 bp (rBRSV/A2).

Fig. 2B shows an analysis of RT-PCR products corresponding to positions 5372 to 5964 (ATue51908 and rBRSV) or positions 5452 to 6062 (rBRSV/A2), containing the G/F intergenic regions and the respective marker restriction sites in the recombinant viruses (an SphI site at rBRSV position 5539 or a StuI site in rBRSV-A2 position 5619). The PCR products were of the expected size of approximately 592 (ATue51908 and rBRSV) or 610 (rBRSV/A2). SphI digested only rBRSV, as predicted, resulting in fragments of 425 bp and 167 bp. StuI digested only rBRSV/A2, as predicted, resulting in fragments of 443 bp and 167 bp.

Fig. 2C shows an analysis of RT-PCR products spanning positions 7218 to 7852 (ATue51908 and rBRSV) or 7316 to 7939 (rBRSV/A2), containing the F/M2 intergenic region. As predicted, digestion with XhoI did not cleave ATue51908 but in the case of rBRSV and rBRSV/A2 resulted in fragments of 395 bp (both viruses) and 267 bp (rBRSV) or 256 bp (rBRSV/A2). This revealed the presence of the introduced XhoI site (rBRSV position 7471, rBRSV-A2 position 7558) in the genomes of the recombinant viruses.

Fig. 3 shows a comparison of the growth of rBRSV, rBRSV/A2 and HRSV strain Long in human HEp-2 cells (Panel A) and bovine MDBK cells (Panel B). Duplicate cell monolayers in 24-well dishes were infected with an MOI of 0.1 of either virus. Supernatant aliquots were taken at the indicated times, frozen at -70°C and titrated later in duplicate. Each value is the mean titer of material from two wells of infected cells.

Fig. 4 provides an electron microscopic examination of virions of recombinant BRSV after fixation with glutardaldehyde, followed by immunogold labeling and negative staining (overview and enlargement) (Panel A), compared to virions detected in ultra thin sections (Panel B). The long filamentous virion shape as well as the antigenic epitopes on the virion surface are conserved by the preparation technique. (Panel A bar: 2 μ , enlargement: bar: 100 nm, Panel B bar: 150 nm).

Fig. 5 shows immunogold labeling using monoclonal antibodies reacting with both HRSV F and BRSV F protein (Panels A, C and E) or exclusively with HRSV F protein (Panels B, D and F). Labeling of preparations of rBRSV (Panels A and B), rBRSV/A2 (Panels C and D), and of HRSV, strain Long (Panels E and F). Bar: 150 nm.

Fig. 6 shows immunogold labeling using monoclonal antibodies reacting with BRSV G protein (Panels A, C and E) or with HRSV G protein (Panels B, D and F). Labeling of preparations of rBRSV (Panels A and B), rBRSV/A2 (Panels C and D), and of HRSV strain Long (Panels E, F). Bar: 150 nm.

Figs. 7A and 7B depict replacement of the F gene of rBRSV strain ATue51908 with the F gene of HRSV strain Long to create the recombinant chimeric virus rBRSV/LongF.

Fig. 7A is a diagram of the rBRSV genome illustrating the replacement of the F gene with that of HRSV strain Long. The location of the open reading frames (ORFs) are shown as shaded (BRSV) or open (HRSV) rectangles. The F gene is shown as an enlargement, in which gene end/polyadenylation signals are represented by bars, gene start signals are shown as filled triangles, and the positions of synthetic restriction sites serving as genetic markers or as boundaries of swapped restriction fragments are indicated by arrowheads with the position in the complete antigenome sequence shown underneath. The size of the fragments framed by the marker restriction sites is indicated (in parentheses). The

genome length of the recombinant viruses is indicated on the left. The roman numerals (I, II and III) refer to intergenic regions which are expanded in Fig. 8B.

Fig. 7B shows alignment of the SH/G (I), G/F (II), and F/M2 (III) intergenic regions of biologically derived BRSV strain ATue51908 (top line); the recombinant version, rBRSV ATue51908 (second line), and the recombinant chimeric virus rBRSV/LongF (bottom line). Annotations are as in Fig. 1. Figure 7B, Panel I: SH/G intergenic region: ATue51908 (top line) (SEQ ID NO. 1); rBRSV (second line) (SEQ ID NO. 2); rBRSV/LongF (third line) (SEQ ID NO. 2). Figure 7B, Panel II: G/F intergenic region: ATue 51908 (top line) (SEQ ID NO. 5); rBRSV (second line) (SEQ ID NO. 6); rBRSV/LongF (third line) (SEQ ID NO. 13). Figure 7B, Panel 3: F/M2 intergenic region: ATue51908 (top line) (SEQ ID NO. 9); rBRSV (second line) (SEQ ID NO. 10); rBRSV/A2 (third line) (SEQ ID NO. 11).

Fig. 8 provides RT-PCR and restriction analysis confirming the identity of the recombinant virus isolate rBRSV/LongF. Two RT-PCRs were done using total RNA from cells infected with indicated virus isolates. The PCR products were analyzed on a 0.8% agarose gel. No PCR product was obtained if the RT step was omitted. Nucleotide lengths are estimates based on calculated sizes. M, 1 kb DNA ladder (Life Technologies, Gaithersburg, MD). The size of some marker fragments is indicated on the left. PCR 1 was done using a primer pair hybridizing with sequences of the BRSV backbone (ATue51908 nt 5380 - 5397 (BRSV G gene), and ATue51908 nt 7567 - 7550 (BRSV M2 gene), upstream and downstream of the F gene. PCR 1 yielded products of 2,187 nt for BRSV strain ATue51908 and the recombinant analog rBRSV, and 2,161 nt for rBRSV/LongF (these bands are not distinguishable by electrophoretic mobility under these conditions). As expected, no PCR product was obtained from HRSV strain Long RNA using the above BRSV primer pair. The products from PCR 1 were subjected to restriction analysis. As predicted, the recombinant viruses, but not standard BRSV strain ATue51908, was cleaved by SphI (yielding bands of 2,028 bp and 159 bp for rBRSV, and 2,002 bp and 159 bp for rBRSV/LongF), demonstrating the existence of the synthetic SphI marker restriction site (rBRSV and rBRSV/LongF genome pos. 5539). EcoRI cleaves at the naturally occurring EcoRI site of the BRSV ATue51908 and the rBRSV F gene (genome pos. 6233) to yield two fragments of 1,334 nt and 853 nt, whereas the fragment originating from rBRSV/LongF is not cleaved by EcoRI, as predicted. When digested by PstI, as expected, the fragments

comprising the BRSV F gene remain uncleaved, and the rBRSV/LongF product is cleaved into three fragments of 1,351 bp, 586 bp, and 224 bp, revealing the existence of two naturally occurring PstI sites (pos. 63 and 649) contained in the HRSV Long F gene. PCR 2 was done using a primer pair hybridizing to the HRSV strain Long F gene (primer FL, 5 3'-GGGGCAAATAACAATGGAGTTGCCAATC-5' (SEQ ID NO. 19), nt 1 -28 of the HRSV strain Long F gene sequence, and primer FLr, 5'-TTTTTATATAACTATAAACTAGGAATCTAC-3' (SEQ ID NO.: 20), HRSV strain Long F gene nt 1903 -1874), yielding a PCR product of 1,903 bp for rBRSV/LongF and the HRSV strain Long parental virus. As expected, no product was obtained from rBRSV. 10 Digestion with EcoRI and PstI yielded the expected cleavage pattern.

Fig. 9 provides an immunoelectron microscopic analysis of the surface proteins of rBRSV/LongF. Panel A: mab G66 to BRSV G protein; Panel B: mab 021/21G to HRSV G protein; Panel C: mab 2F, reactive with F proteins of both HRSV and BRSV; and Panel D: mab 44F, specific for HRSV F protein. bar: 150 nm.

15 Fig. 10 details construction of a chimeric rBRSV/HRSV genome in which the BRSV G and F genes have been deleted and the G and F genes of HRSV have been placed in a promoter-proximal position. BRSV genes are shaded; HRSV genes are clear. Nucleotide sequence position numbers are relative to the complete rBRSV antigenome (Buchholz et al., *J. Virol.* 73:251-259, 1999; Buchholz et al., *J. Virol.* 74:1187-1199, 2000; 20 GenBank accession number AF092942 or complete rHRSV antigenome in Collins et al., *Proc. Natl. Acad. Sci. USA* 92:11563-11567, 1995; each incorporated herein by reference); sequence position numbers that refer to the HRSV sequence are underlined. Fig. 10, panel A details structure of rBRSV containing *NotI*, *SalI* and *XhoI* sites that were added in previous work (Buchholz et al., *J. Virol.* 73:251-259, 1999; Buchholz et al., *J. Virol.* 74:1187-1199, 25 2000). Fig. 10, panel B depicts modifications to rBRSV to create rBRSV/A2-G1F2. The BRSV G and F genes were deleted by digestion with *SalI* and *XhoI* and religation of the resulting compatible cohesive ends. The region of the genome of HRSV from nucleotides 4692 to 7557, containing the G and F genes, was amplified by PCR using primers that contained desired changes to be incorporated into each end of the cDNA. The amplified 30 PCR product contained (in upstream to downstream order): a *NotI* site, a *BspI* site, the complete HRSV G ORF, its downstream noncoding and GE signal, the HRSV G-F IG sequence, the complete HRSV F gene, 6 nucleotides from the HRSV F-M2 IG sequence

(CACAAT), the NS1 GS signal, a *BlnI* site and a *NotI* site. This cDNA was cloned into the unique *NotI* site at position 67 of rBRSV. Fig. 10, panel C illustrates structure of the genomic RNA of rBRSV/A2-G1 F2.

Fig. 11 depicts multicycle growth of RBRV, rHRSV(rA2), rBRSV/A2, and rBRSV/A2-G1 F2 in HEp-2 human (left panel) and MDBK bovine (right panel) cells. Duplicate cell monolayers were infected with the indicated virus at an MOI of 0.1 and incubated at 37°C, and medium aliquots were harvested at the indicated times, flash frozen, stored at -70°C and titrated later in duplicate. Each value is the mean titer of two wells.

Fig. 12. shows indirect immunofluorescence of HEp-2 cells infected with rBRSV/A2-G1F2, rBRSV/A2, or rA2. Cells were infected at an MOI of 0.1, incubated at 37°C for 96 hours, fixed with acetone, permeabilized and reacted with monoclonal antibody 021/1G, specific to the G protein of HRSV, or with monoclonal antibody 44F, specific to the F protein of HRSV. Antibody binding was visualized by reaction with a tagged antibody specific to murine IgG.

Fig. 13 details construction of a chimeric rBRSV/HRSV containing the M, G and F genes of HRSV. BRSV genes are shaded; HRSV genes are clear. Sequence position numbers that refer to HRSV genes are underlined. Fig. 13, panel A depicts modification of rBRSV/A2 to contain a unique *MluI* site at position 3204, within the intergenic region between the P and M genes (P-M IG). The sequence of the IG is shown, with small case letters indicating the original nucleotide assignments. The underlined letters indicate the *MluI* site created by the 5 nucleotide substitutions. Nucleotide sequence position numbers are relative to the complete rBRSV antigenome (Buchholz et al., *J. Virol.* 73:251-259, 1999; Buchholz et al., *J. Virol.* 74:1187-1199, 2000; GenBank accession number AF092942 or complete rHRSV antigenome in Collins et al., *Proc. Natl. Acad. Sci. USA* 92:11563-11567, 1995); sequence position numbers that refer to the HRSV sequence are underlined. Fig. 13, panel B illustrates modification of rBRSV/A2 to create rBRSV/A2-MGF. The *MluI*-*SaII* fragment containing the BRSV M and SH genes was excised and replaced with an *MluI*-*SaII* fragment containing the HRSV M gene. The *MluI*-*SaII* fragment containing the HRSV M gene is shown in the box. Also shown is the sequence immediately upstream of the *MluI* site (SEQ ID NO. 17), including the BRSV P gene-end sequence, and the sequence immediately downstream of the *SaII* site (SEQ ID NO. 18), including the intergenic sequence between the M and G genes (M-G IG), the HRSV G gene-start signal, and the ATG (bold, italics) that

begins the G ORF. Fig., 13, panel C depicts the structure of the genome of rBRSV/A2-MGF.

DESCRIPTION OF THE SPECIFIC EMBODIMENTS

5 The present invention provides recombinant respiratory syncytial virus (RSV) cloned as a chimera of human and bovine RSV genomic or antigenomic sequences to yield a human-bovine chimeric RSV. The chimeric construction of human-bovine RSV yields a viral particle or subviral particle that is infectious in mammals, particularly humans, and useful for generating immunogenic compositions for clinical use. Also provided within the
10 invention are novel methods and compositions for designing and producing attenuated, human-bovine chimeric RSV, as well as methods and compositions for the prophylaxis and treatment of RSV infection. Human-bovine chimeric RSV and immunogenic compositions according to the invention may elicit an immune response to a specific RSV subgroup or strain, or they may elicit a polyspecific response against multiple RSV subgroups or strains.

15 RSV is generally characterized as an enveloped nonsegmented negative strand RNA virus of the paramyxovirus family (Collins, et al., Fields Virology 2:1313-1352, 1996). Its genome, which is 15,222 nucleotides (nt) in length for the well known strain A2, is transcribed into 10 messenger RNAs that encode 11 proteins (Collins, et al., Fields Virology 2:1313-1352, 1996; Atreya, et al., J. Virol. 72:1452-61, 1998; Bukreyev, et al., J. Virol. 71:8973-82, 1997; Collins, et al., Proc. Natl. Acad. Sci. USA 93:81-5, 1996; Teng and
20 Collins, J. Virol. 72:5707-16, 1998; Teng and Collins, J. Virol. 73:466-473, 1999; Whitehead, et al., J. Virol. 73:3438-42, 1999, each incorporated herein by reference). Four of these are nucleocapsid/polymerase proteins, namely the major nucleocapsid N protein, the phosphoprotein P, and polymerase protein L, and the transcription antitermination protein
25 M2-1. Three are surface glycoproteins, namely the attachment G protein, the fusion F glycoprotein responsible for penetration and syncytium formation, and the small hydrophobic SH protein of unknown function. The matrix M protein is an internal virion protein involved in virion formation. There are two nonstructural proteins NS1 and NS2 of unknown function. Finally, there is a second open reading frame (ORF) in the M2 mRNA
30 which encodes an RNA regulatory factor M2-2.

Gen. Virol. 73:737-741, 1992; Mallipeddi and Samal, J. Gen. Virol. 73:2441-2444, 1992, Pastey and Samal, J. Gen. Virol. 76:193-197, 1995; Walravens et al., J. Gen. Virol. 71:3009-3014, 1990; Yunnus et al., J. Gen. Virol. 79:2231-2238, 1998, each incorporated herein by reference). The ability to produce infectious HRSV from cDNA has been extended to allow
5 recombinant production of BRSV, and infectious recombinant virus has thereby been produced (Buchholz, et al., J. Virol. 73:251-9, 1999).

BRSV has been evaluated in cotton rats (Piazza, et al., J. Virol. 67:1503-10, 1993), owl monkeys and chimpanzees (Prince, et al., Animal Models of Respiratory Syncytial Virus Infections, 1990) as a vaccine against HRSV. BRSV was highly restricted
10 in all three experimental animals, and immune responses were very low. In rats, significant resistance to HRSV challenge was observed in one study, but the basis of this protective effect remains unclear. In monkeys, antibody responses to the BRSV vaccine were detected in only 2 of 9 animals. Upon subsequent challenge with HRSV, the duration of shedding was slightly reduced, but neither the magnitude of shedding nor disease symptoms were
15 affected. In a parallel study of two chimpanzees which received a high inoculum of BRSV, there was evidence of replication in only one animal and, upon subsequent challenge with HRSV, the duration of shedding was slightly reduced but otherwise the titer of HRSV and the magnitude of clinical symptoms were undiminished. These studies indicate that isolates of BRSV found in nature are not suited for direct vaccine use.

The instant invention provides for development of live-attenuated RSV
20 vaccine candidates based on chimeras between HRSV and BRSV. Chimeras are constructed through a cDNA intermediate and cDNA-based recovery system. Recombinant viruses which are made from cDNA replicate independently and are propagated in the same manner as if they were biologically-derived, and indeed can only be distinguished from biologically
25 derived viruses by the presence of mutations which have been specifically inserted. Chimeric HRSV/BRSV vaccine candidates preferably bear one or more of the major antigenic determinants of HRSV in a background which is attenuated by the presence of one or more BRSV genes. In addition, human-bovine chimeric RSV according to the invention can be further modified to incorporate specific attenuating mutations, as well as a variety of
30 other mutations and nucleotide modifications, to yield desired structural or phenotypic affects.

Detailed descriptions of the materials and methods for producing recombinant

RSV from cDNA, and for making and testing the full range of mutations and nucleotide modifications disclosed herein as supplemental aspects of the present invention, are set forth in, e.g., U.S. Provisional Patent Application No. 60/007,083, filed September 27, 1995; U.S. Patent Application No. 08/720,132, filed September 27, 1996; U.S. Provisional Patent Application No. 60/021,773, filed July 15, 1996; U.S. Provisional Patent Application No. 60/046,141, filed May 9, 1997; U.S. Provisional Patent Application No. 60/047,634, filed May 23, 1997; U.S. Patent No. 5,993,824, issued November 30, 1999 (corresponding to International Publication No. WO 98/02530); U.S. Patent Application No. 09/291,894, filed by Collins et al. on April 13, 1999; U.S. Provisional Patent Application No. 60/129,006, filed by Murphy et al. on April 13, 1999; Crowe et al., Vaccine 12: 691-699, 1994; and Crowe et al., Vaccine 12: 783-790, 1994; Collins, et al., Proc Nat. Acad. Sci. USA 92:11563-11567, 1995; Bukreyev, et al., J Virol 70:6634-41, 1996; Juhasz et al., J. Virol. 71(8):5814-5819, 1997; Durbin et al., Virology 235:323-332, 1997; Karron et al., J. Infect. Dis. 176:1428-1436, 1997); He et al. Virology 237:249-260, 1997; Baron et al. J. Virol. 71:1265-1271, 1997; Whitehead et al., Virology 247(2):232-9, 1998a; Whitehead et al., J. Virol. 72(5):4467-4471, 1998b; Jin et al. Virology 251:206-214, 1998; Bukreyev, et al., Proc. Nat. Acad. Sci. USA 96:2367-2372, 1999; Bermingham and Collins, Proc. Natl. Acad. Sci. USA 96:11259-11264, 1999 Juhasz et al., Vaccine 17:1416-1424, 1999; Juhasz et al., J. Virol. 73:5176-5180, 1999; Teng and Collins, J. Virol. 73:466-473, 1999; Whitehead et al., J. Virol. 73:9773-9780, 1999; Whitehead et al., J. Virol. 73:871-877, 1999; and Whitehead et al., J. Virol. 73:3438-3442, 1999. Exemplary methods for producing recombinant RSV from cDNA involve intracellular coexpression, typically from plasmids cotransfected into tissue culture cells, of an RSV antigenomic RNA and the RSV N, P, M2-1 and L proteins. This launches a productive infection that results in the production of infectious cDNA-derived virus, which is termed recombinant virus. Once generated, recombinant RSV is readily propagated in the same manner as biologically-derived virus, and a recombinant virus and a counterpart biologically-derived virus cannot be distinguished unless the former had been modified to contain one or more introduced changes as markers.

In more detailed aspects, the foregoing incorporated documents describe methods and procedures useful within the invention for mutagenizing, isolating and characterizing RSV to obtain attenuated mutant strains (e.g., temperature sensitive (ts), cold passaged (cp) cold-adapted (ca), small plaque (sp) and host-range restricted (hr) mutant strains) and for identifying the genetic changes that specify the attenuated phenotype. In

conjunction with these methods, the foregoing documents detail procedures for determining replication, immunogenicity, genetic stability and protective efficacy of biologically derived and recombinantly produced attenuated human RSV, including human RSV A and B subgroups, in accepted model systems, including murine and non-human primate model systems. In addition, these documents describe general methods for developing and testing immunogenic compositions, including monovalent and bivalent vaccines, for prophylaxis and treatment of RSV infection.

The ability to generate infectious RSV from cDNA provides a method for introducing predetermined changes into infectious virus via the cDNA intermediate. This method has been demonstrated to produce a wide range of infectious, attenuated derivatives of RSV, for example recombinant vaccine candidates containing one or more amino acid substitutions in a viral protein, deletion of one or more genes or ablation of gene expression, and/or one or more nucleotide substitutions in cis-acting RNA signals yielding desired effects on viral phenotype (see, e.g., Bukreyev et al., J. Virol. 71:8973-8982, 1997; Whitehead et al., J. Virol. 72:4467-4471, 1998; Whitehead et al., Virology 247:232-239, 1998; Bermingham and Collins, Proc. Natl. Acad. Sci. USA 96:11259-11264, 1999; Juhasz et al., Vaccine 17:1416-1424, 1999; Juhasz et al., J. Virol. 73:5176-5180, 1999; Teng and Collins, J. Virol. 73:466-473, 1999; Whitehead et al., J. Virol. 73:871-877, 1999; Whitehead et al., J. Virol. 73:3438-3442, 1999; and Collins et al., Adv. Virus Res. 54:423-451, 1999, each incorporated herein by reference).

Exemplary of the foregoing teachings are methods for constructing and evaluating infectious recombinant RSV modified to incorporate phenotype-specific mutations identified in biologically-derived RSV mutants, e.g., cp and ts mutations adopted in recombinant RSV from biologically derived designated cpts RSV 248 (ATCC VR 2450), cpts RSV 248/404 (ATCC VR 2454), cpts RSV 248/955 (ATCC VR 2453), cpts RSV 530 (ATCC VR 2452), cpts RSV 530/1009 (ATCC VR 2451), cpts RSV 530/1030 (ATCC VR 2455), RSV B-1 cp52/2B5 (ATCC VR 2542), and RSV B-1 cp-23 (ATCC VR 2579). These methods are readily adapted for construction of recombinant human-bovine chimeric RSV of the invention. The recombinant RSV thus provided may incorporate two or more ts mutations from the same, or different, biologically derived RSV mutant(s), for example one or more of the 248/404, 248/955, 530/1009, or 530/1030 biological mutants. In the latter context, multiply attenuated recombinants may have a combination of attenuating mutations

from two, three or more biological mutants, e.g., a combination of attenuating mutations from the RSV mutants 530/1009/404, 248/404/1009, 248/404/1030, or 248/404/1009/1030 mutants. In exemplary embodiments, one or more attenuating mutations specify a temperature-sensitive substitution at amino acid Asn43, Phe521, Gln831, Met1169, or

5 Tyr1321 in the RSV polymerase gene or a temperature-sensitive nucleotide substitution in the gene-start sequence of gene M2. Preferably, these mutations involve identical or conservative changes with the following changes identified in biologically derived mutant RSV, for example changes conservative to the following substitutions identified in the L polymerase gene: Ile for Asn43, Leu for Phe521, Leu for Gln831, Val for Met1169, and Asn
10 for Tyr1321.

Yet additional mutations that may be incorporated in human-bovine chimeric RSV of the invention are mutations, e.g., attenuating mutations, identified in heterologous RSV or more distantly related negative stranded RNA viruses. In particular, attenuating and other desired mutations identified in one negative stranded RNA virus may be “transferred”, e.g., copied, to a corresponding position within a human or bovine RSV genome or antigenome, either within the human-bovine chimeric RSV or as a means of constructing the human-bovine chimeric RSV. Briefly, desired mutations in one heterologous negative stranded RNA virus are transferred to the RSV recipient (e.g., bovine or human RSV, respectively). This involves mapping the mutation in the heterologous virus, thus identifying by sequence alignment the corresponding site in human or bovine RSV, and mutating the native sequence in the RSV recipient to the mutant genotype (either by an identical or conservative mutation), as described in U.S. Provisional Patent Application No. 60/129,006, filed by Murphy et al. on April 13, 1999, incorporated herein by reference. As this disclosure teaches, it is preferable to modify the chimeric genome or antigenome to encode an alteration at the subject site of mutation that corresponds conservatively to the alteration identified in the heterologous mutant virus. For example, if an amino acid substitution marks a site of mutation in the mutant virus compared to the corresponding wild-type sequence, then a similar substitution should be engineered at the corresponding residue(s) in the recombinant virus. Preferably the substitution will involve an identical or conservative amino acid to the substitute residue present in the mutant viral protein. However, it is also possible to alter the native amino acid residue at the site of mutation non-conservatively with respect to the substitute residue in the mutant protein (e.g., by using any other amino acid to disrupt or impair the function of the wild-type residue). Negative

in which expression of the SH gene was been ablated by removal of a polynucleotide sequence encoding the SH mRNA and protein. Deletion of the SH gene yielded not only recoverable, infectious RSV, but one which exhibited substantially improved growth in tissue culture based on both yield of infectious virus and plaque size. This improved growth in tissue culture specified by the SH deletion provides useful tools for developing human-bovine chimeric RSV vaccines, for example by overcoming problems of poor RSV yields in culture. Moreover, these deletions are highly stable against genetic reversion, rendering RSV clones derived therefrom particularly useful as vaccine agents.

SH-minus RSV recombinants also exhibit site-specific attenuation in the upper respiratory tract of mice, which presents novel advantages for vaccine development. Current RSV strains under evaluation as live virus vaccines, for example cp mutants, do not exhibit significantly altered growth in tissue culture. These are host range mutations and they restrict replication in the respiratory tract of chimpanzees and humans approximately 100-fold in the lower respiratory tract. Another exemplary type of mutation, ts mutations, tend to preferentially restrict virus replication in the lower respiratory tract due to the gradient of increasing body temperature from the upper to the lower respiratory tract. In contrast to these cp and ts mutants, SH-minus RSV mutants have distinct phenotypes of greater restriction in the upper respiratory tract. This is particularly desirable for vaccine viruses for use in very young infants, because restriction of replication in the upper respiratory tract is required to ensure safe vaccine administration in this vulnerable age group whose members breathe predominantly through the nose. Further, in any age group, reduced replication in the upper respiratory tract will reduce morbidity from otitis media. In addition to these advantages, the nature of SH deletion mutations, involving e.g., nearly 400 nt and ablation of an entire mRNA, represents a type of mutation which will be highly refractory to reversion.

Also discussed in the context of SH gene modifications is a comparison of SH genes among different RSVs, including human and bovine RSVs, and other pneumoviruses to provide additional tools and methods for generating useful RSV recombinant vaccines. For example, the two RSV antigenic subgroups, A and B, exhibit a relatively high degree of conservation in certain SH domains. In two such domains, the N-terminal region and putative membrane-spanning domains of RSV A and B display 84% identity at the amino acid level, while the C-terminal putative ectodomains are more divergent (approx. 50%

identity). Comparison of the SH genes of two human RSV subgroup B strains, 8/60 and 18537, identified only a single amino acid difference (Anderson et al., supra). The SH proteins of human versus bovine RSV are approximately 40% identical, and share major structural features including (i) an asymmetric distribution of conserved residues; (ii) very similar hydrophobicity profiles; (iii) the presence of two N-linked glycosylation sites with one site being on each side of the hydrophobic region; and (iv) a single cysteine residue on the carboxyterminal side of the central hydrophobic region of each SH protein. (Anderson et al., supra). By evaluating these and other sequence similarities and differences, selections can be made of heterologous sequence(s) that can be substituted or inserted within infectious human-bovine chimeric RSV clones, for example to yield vaccines having multi-specific immunogenic effects or, alternatively or in addition, desirable effects such as attenuation.

Also disclosed in the context of gene deletions are the effects of changing gene position. For example, deletion of the SH gene results in an effective change in downstream gene position to a more promoter proximal position. This may be associated with an increase in transcription of downstream genes in the recombinant virus. Thus, methods are provided for altering levels of RSV gene expression by changing gene order or position in the genome or antigenome. Decreased levels of expression of downstream genes are expected to specify attenuation phenotypes, whereas increased expression can achieve the opposite effects in recombinant RSV in permissive hosts, e.g., chimpanzees and humans.

In another example described in the above-incorporated references, expression of the NS2 gene is ablated by introduction of stop codons into the translational open reading frame (ORF). The rate of release of infectious virus was reduced for this NS2 knock-out virus compared to wild-type. In addition, comparison of the plaques of the mutant and wild-type viruses showed that those of the NS2 knock-out were greatly reduced in size. This type of mutation can thus be incorporated within viable recombinant human-bovine chimeric RSV to yield altered phenotypes, in this case reduced rate of virus growth and reduced plaque size *in vitro*. These and other knock-out methods and mutants will therefore provide for yet additional recombinant RSV vaccine agents, based on the known correlation between reduced plaque size *in vitro* and attenuation *in vivo*. Expression of the NS2 gene also was ablated by complete removal of the NS2 gene, yielding a virus with a similar phenotype.

Other RSV genes which have been successfully deleted include the NS1 and

Th2-biased response, which in turn appears to be associated with enhanced immunopathology upon subsequent exposure to RSV. With regard to an RSV vaccine virus, it is highly desirable to minimize antibody trapping or imbalanced stimulation of the immune system, and so it would be desirable to ablate expression of the secreted form of the G protein. This has been achieved in recombinant virus. Thus, this mutation is particularly useful to qualitatively and/or quantitatively alter the host immune response elicited by the recombinant virus, rather than to directly attenuate the virus.

The incorporated references also describe modulation of the phenotype of recombinant RSV by altering cis-acting transcription signals of exemplary genes, e.g., NS1 and NS2. The results of these nucleotide modifications are consistent with modification of gene expression by altering cis-regulatory elements, for example to decrease levels of readthrough mRNAs and increase expression of proteins from downstream genes. The resulting recombinant viruses will preferably exhibit increased growth kinetics and increased plaque size. Exemplary modifications to cis-acting regulatory sequences include modifications to gene end (GE) and gene start (GS) signals associated with RSV genes. In this context, exemplary changes include alterations of the GE signals of the NS1 and NS2 genes rendering these signals identical to the naturally-occurring GE signal of the RSV N gene. The resulting recombinant virus exhibits increased growth kinetics and plaque size and therefore provide yet additional means for beneficially modifying phenotypes of human-bovine chimeric RSV vaccine candidates.

Chimeric bovine-human RSV formed of a partial or complete BRSV background genome or antigenome and a heterologous gene or genome segment from HRSV may incorporate a heterologous gene or genome segment from a subgroup A or subgroup B HRSV, from both subgroup A and B HRSV, or from one or more specific HRSV strains. Exemplary HRSV strains in this context include the well known strains RSV A2 and Long. In preferred aspects, one or more HRSV glycoprotein genes F, G and SH, or a genome segment encoding a cytoplasmic domain, transmembrane domain, ectodomain or immunogenic epitope of a HRSV glycoprotein gene is substituted for a counterpart gene or genome segment within the BRSV background genome or antigenome. In alternate embodiments one or both of the HRSV glycoprotein genes F and G is/are substituted to replace one or both counterpart F and G glycoprotein genes in the BRSV background genome or antigenome. In one specific example described herein below, the F gene from

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HRSV Long is substituted into the rBRSV strain ATue51908 in place of the counterpart BRSV F gene. In another example, both HRSV glycoprotein genes F and G of the HRSV strain A2 are substituted to replace counterpart F and G glycoprotein genes in the BRSV ATue51908 strain background genome or antigenome. In alternative embodiments,
5 chimeric human-bovine RSV of the invention incorporate antigenic determinants from more than one RSV strain or subgroup, e.g., both human RSV subgroup A and subgroup B.

Thus, methods and compositions provided in the above-incorporated references that allow production of attenuated human-bovine chimeric RSV vaccine viruses comprising sequences from both RSV subgroups A and B, e.g., to yield a RSV A or B
10 vaccine or a bivalent RSV A/B vaccine (see, e.g., U.S. Patent Application No. 09/291,894, filed by Collins et al. on April 13, 1999, incorporated herein by reference). In this context, a human-bovine chimeric RSV may comprise a BRSV into which multiple HRSV gene(s) or genome segment(s) are introduced encoding antigenic determinants from both HRSV A and HRSV B subgroups. This virus can then be attenuated to a desired level by systematic
15 incorporation of attenuating mutations as described above. For example, specific attenuating mutations that have been incorporated into chimeric RSV A/B viruses include: (i) three of the five cp mutations, namely the mutation in N (V267I) and the two in L (C319Y and H1690Y), but not the two in F since these are removed by substitution with the B1 F gene; (ii) the 248 (Q831L), 1030 (Y1321N) and, optionally, 404-L (D1183E) mutations which
20 have been identified in attenuated strain A2 viruses; (iii) the single nucleotide substitution at position 9 in the gene-start signal of the M2 gene, and (iv) deletion of the SH gene. Other immediately available mutations in human-bovine chimeric RSV carrying RSV A and or RSV B genes or genome segments include, but are not limited to, NS1, NS2, G, or M2-2 gene deletions, and the 530 and 1009 mutations, alone or in combination.

25 In other detailed aspects of the invention, human-bovine chimeric RSV are employed as "vectors" for protective antigens of heterologous pathogens, including other RSVs and non-RSV viruses and non-viral pathogens. Within these aspects, the bovine-human chimeric genome or antigenome comprises a partial or complete RSV "vector genome or antigenome" combined with one or more heterologous genes or genome
30 segments encoding one or more antigenic determinants of one or more heterologous pathogens (see, e.g., U.S. Provisional Patent Application Serial No. 60/170,195; U.S. Patent Application No. 09/458,813; and U.S. Patent Application No. 09/459,062, each incorporated

herein by reference). The heterologous pathogen in this context may be a heterologous RSV (e.g., a different RSV strain or subgroup) and the heterologous gene(s) or genome segment(s) can be selected to encode one or more of the above identified RSV proteins, as well as protein domains, fragments, and immunogenic regions or epitopes thereof. RSV vector vaccines thus constructed may elicit a polyspecific immune response and may be administered simultaneously or in a coordinate administration protocol with other vaccine agents.

Human-bovine chimeric RSV engineered as vectors for other pathogens may comprise a vector genome or antigenome that is a partial or complete HRSV genome or antigenome, which is combined with or is modified to incorporate one or more heterologous genes or genome segments encoding antigenic determinant(s) of one or more heterologous RSV(s), including heterologous HRSVs selected from HRSV A or HRSV B. In alternative aspects, the vector genome or antigenome is a partial or complete HRSV genome or antigenome and the heterologous gene(s) or genome segment(s) encoding the antigenic determinant(s) is/are of one or more non-RSV pathogens. Typically, the chimeric genome or antigenome incorporates one or more gene(s) or genome segment(s) of a BRSV that specifies attenuation. Alternatively, the vector virus may be comprise a partial or complete BRSV background genome or antigenome incorporating one or more HRSV genes or genome segments, wherein the human-bovine chimeric RSV vector virus is modified to include one or more donor gene(s) or genome segment(s) encoding an antigenic determinant of a non-RSV pathogen.

Thus, in certain detailed aspects of the invention, human-bovine chimeric RSV are provided as vectors for a range of non-RSV pathogens (see, e.g., U.S. Provisional Patent Application Serial No. 60/170,195; U.S. Patent Application No. 09/458,813; and U.S. Patent Application No. 09/459,062, each incorporated herein by reference). The vector genome or antigenome for use within these aspects of the invention may comprise a partial or complete BRSV or HRSV genome or antigenome incorporating, respectively, a heterologous HRSV or BRSV gene or genome segment, and the heterologous pathogen may be selected from measles virus, subgroup A and subgroup B respiratory syncytial viruses, HPIV1, HPIV2, HPIV3, mumps virus, human papilloma viruses, type 1 and type 2 human immunodeficiency viruses, herpes simplex viruses, cytomegalovirus, rabies virus, Epstein Barr virus, filoviruses, bunyaviruses, flaviviruses, alphaviruses and influenza viruses.

For example, a HRSV or BRSV vector genome or antigenome for constructing bovine-human chimeric RSV of the invention may incorporate heterologous antigenic determinant(s) selected from the measles virus HA and F proteins, or antigenic domains, fragments and epitopes thereof. In exemplary embodiments, a transcription unit comprising an open reading frame (ORF) of a measles virus HA gene is added to or incorporated within a BRSV or HRSV3 vector genome or antigenome. Alternatively bovine-human chimeric RSV of the invention may be used as vectors to incorporate heterologous antigenic determinant(s) from a parainfluenza virus (PIV), for example by incorporating one or more genes or genome segments that encode(s) a HPIV1, HPIV2, or HPIV3 HN or F glycoprotein or immunogenic domain(s) or epitope(s) thereof.

As noted above, exemplary embodiments of the invention feature replacement or addition of a heterologous gene(s), genome segment(s), or single or multiple nucleotides of a human or bovine RSV, which are added to or substituted within a partial or complete background bovine or human RSV genome or antigenome to produce the human-bovine chimeric RSV genome or antigenome. In one aspect of the invention, human-bovine chimeric RSV incorporate a partial or complete bovine background RSV genome or antigenome combined with one or more heterologous gene or genome segment from one or more human RSV subgroup(s) or strain(s). Alternatively, human-bovine chimeric RSV may incorporate a partial or complete human background RSV genome or antigenome combined with a heterologous gene or genome segment from one or more bovine RSV. Genes and genome segments that are selected for use as heterologous inserts or additions to the background genome or antigenome include any wild-type or mutant genes or genome segments encoding an RSV NS1, NS2, N, P, M, SH, M2(ORF1), M2(ORF2), L, F or G proteins, or a portion thereof or any additional genome segments. Preferably, the heterologous human gene(s) encode(s) an RSV F, G or SH glycoprotein to provide desired immunogenic effects. In one exemplary embodiment, one or more human RSV glycoprotein genes F, SH, and/or G are added to or substituted within a partial or complete bovine genome or antigenome to yield an attenuated, infectious human-bovine chimera that elicits an anti-human RSV immune response in a susceptible host. Alternatively, the human-bovine chimeric RSV may incorporate one or more genome segment(s) encoding a cytoplasmic domain, transmembrane domain, ectodomain or immunogenic epitope of a human RSV F, G or SH glycoprotein. These immunogenic proteins, domains and epitopes also generate novel immune responses in an immunized host. For example, addition or

In other embodiments, human-bovine chimeric RSV useful in a vaccine formulation can be conveniently modified to accommodate antigenic drift in circulating virus. Typically the modification will be in the G and/or F proteins. An entire G or F gene, or a genome segment encoding a particular immunogenic region thereof, from one RSV strain is incorporated into a chimeric RSV genome or antigenome cDNA by replacement of a corresponding region in a recipient clone of a different RSV strain or subgroup, or by adding one or more copies of the gene, such that several antigenic forms are represented. Progeny virus produced from the modified RSV clone can then be used in vaccination protocols against emerging RSV strains.

A variety of additional embodiments of the invention involve the addition or substitution of only a portion of a donor gene of interest to the recipient genome or antigenome. Commonly, non-coding nucleotides such as cis-acting regulatory elements and intergenic sequences need not be transferred with the donor gene coding region. Thus, a coding sequence (e.g., a partial or complete open reading frame (ORF)) of a particular gene may be added or substituted to the partial or complete background genome or antigenome under control of a heterologous promoter (e.g., a promoter existing in the background genome or antigenome) of a counterpart gene or different gene as compared to the donor sequence. A variety of additional genome segments provide useful donor polynucleotides for inclusion within a chimeric genome or antigenome to express chimeric RSV having novel and useful properties. For example, heterologous genome segments may encode part or all of a glycoprotein cytoplasmic tail region, transmembrane domain or ectodomain, an epitopic site or region, a binding site or region containing a binding site, an active site or region containing an active site, etc., of a selected protein from a human or bovine RSV. These and other genome segments can be added to a complete background genome or antigenome or substituted therein for a counterpart genome segment to yield novel chimeric RSV recombinants. Certain recombinants will express a chimeric protein, e.g., a protein having a cytoplasmic tail and/or transmembrane domain of one RSV fused to an ectodomain of another RSV.

According to one aspect of the invention, human-bovine chimeric RSV are constructed by substituting the heterologous gene or genome segment for a counterpart gene or genome segment in a partial RSV background genome or antigenome. Alternatively, the heterologous gene or genome segment may be added as a supernumerary gene or genome

segment in combination with a complete (or partial if another gene or genome segment is deleted) RSV background genome or antigenome. The heterologous gene or genome segment may be added at an intergenic position within a partial or complete RSV background genome or antigenome so as not to disrupt an open reading frame within the background genome or antigenome.

The heterologous gene or genome segment may be added or substituted at a position corresponding to a wild-type gene order position of a counterpart gene or genome segment within the partial or complete RSV background genome or antigenome, which counterpart gene or genome segment is thereby replaced or displaced (e.g., to a more promoter-distal position). In yet additional embodiments, the heterologous gene or genome segment is added or substituted at a position that is more promoter-proximal or promoter-distal compared to a wild-type gene order position of the counterpart gene or genome segment within the background genome or antigenome, which enhances or reduces, respectively, expression of the heterologous gene or genome segment.

With regard to gene order, a number of the foregoing incorporated references have focused on modification of the naturally-occurring order in RSV and other viruses. For example, in RSV the NS1, NS2, SH and G genes were deleted individually, and the NS1 and NS2 gene were deleted together, thereby shifting the position of each downstream gene relative to the viral promoter. For example, when NS1 and NS2 are deleted together, N is moved from position 3 to position 1, P from position 4 to position 2, and so on. Alternatively, deletion of any other gene within the gene order will affect the position (relative to the promoter) only of those genes which are located further downstream. For example, SH occupies position 6 in wild type virus, and its deletion does not affect M at position 5 (or any other upstream gene) but moves G from position 7 to 6 relative to the promoter. It should be noted that gene deletion also can occur (rarely) in a biologically-derived mutant virus. For example, a subgroup B RSV that had been passaged extensively in cell culture spontaneously deleted the SH and G genes (Karron et al., Proc. Natl. Acad. Sci. USA 94:13961-13966, 1997; incorporated herein by reference). Note that "upstream" and "downstream" refer to the promoter-proximal and promoter-distal directions, respectively (the promoter is at the 3' leader end of negative-sense genomic RNA).

Gene order shifting modifications (i.e., positional modifications moving one or more genes to a more promoter-proximal or promoter-distal location in the recombinant

viral genome) with human-bovine chimeric RSV of the invention result in viruses with altered biological properties. For example, RSV lacking NS1, NS2, SH, G, NS1 and NS2 together, or SH and G together, have been show to be attenuated *in vitro*, *in vivo*, or both. It is likely that this phenotype was due primarily to the loss of expression of the specific viral protein. However, the altered gene map also likely contributed to the observed phenotype. This effect is well-illustrated by the SH-deletion virus, which grew more efficiently than wild type in some cell types, probably due to an increase in the efficiency of transcription, replication or both resulting from the gene deletion and resulting change in gene order and possibly genome size. In other viruses, such as RSV in which NS1 and/or NS2 were deleted, altered growth that might have occurred due to the change in gene order likely was obscured by the more dominant phenotype due to the loss of expression of the RSV protein(s).

Yet additional changes have been introduced to change the gene order of RSV in an effort to improve its properties as a live-attenuated vaccine. (see, U.S. Provisional Patent Application entitled RESPIRATORY SYNCYTIAL VIRUS VACCINES EXPRESSING PROTECTIVE ANTIGENS FROM PROMOTOR-PROXIMAL GENES, filed by Kreml et al., June 23, 2000 and identified by Attorney Docket Number 015280-424000US, incorporated herein by reference). In particular, the G and F genes were shifted, singly and in tandem, to a more promoter-proximal position relative to their wild-type gene order. These two proteins normally occupy positions 7 (G) and 8 (F) in the RSV gene order (NS1-NS2-N-P-M-SH-G-F-M2-L). In order to increase the possibility of successful recovery, the manipulations were performed in a version of RSV in which the SH gene had been deleted (Whitehead et al., J. Virol., 73:3438-42 (1999), incorporated herein by reference). This facilitates recovery because this virus makes larger plaques *in vitro* (Bukreyev et al., J. Virol., 71:8973-82 (1997), incorporated herein by reference). G and F were then moved individually to position 1, or were moved together to positions 1 and 2, respectively.

Surprisingly, recombinant RSV were readily recovered in which G or F were moved to position 1, or in which G and F were moved to positions 1 and 2, respectively. This result differed greatly from previous studies with VSV, where movement of the single VSV glycoprotein gene by only two positions was very deleterious to virus growth. The ability to recover these altered viruses also was surprising because RSV replicates inefficiently and because RSV has a complex gene order and movement of the glycoprotein

promoter, namely gene positions 7 and 8, respectively, to positions 1 and 2, respectively.

The resulting chimeric recombinant virus, rBRSV/A2-G1F2, was very similar in its levels of F and G protein expression as detected by immunofluorescence to that of wt HRSV, which result is consistent with increased expression of the G and F glycoproteins attributed to the promoter-proximal shift of the genes. Since the present rBRSV/A2-G1F2 virus bears the same constellation of BRSV genes in its genetic background, it is likely to share this strong host range restriction phenotype. In this context, the increased expression of the two protective antigens in vivo will increase the immunogenicity of this virus to produce highly desirable vaccine properties.

To construct chimeric RSV, heterologous genes may be added or substituted in whole or in part to the background genome or antigenome. In the case of chimeras generated by substitution, a selected gene or genome segment encoding a protein or protein region (e.g., a cytoplasmic tail, transmembrane domain or ectodomain, an epitopic site or region, a binding site or region, an active site or region containing an active site, etc.) from a human or bovine RSV is substituted for a counterpart gene or genome segment in the background RSV genome or antigenome to yield novel recombinants having desired phenotypic changes compared to one or both of the respective wild-type (or mutant parent) RSV strains. As used herein, "counterpart" genes or genome segments refer to counterpart polynucleotides from different RSV sources that encode homologous or equivalent proteins or protein domains, epitopes, or amino acid residues, or which represent homologous or equivalent cis-acting signals which may include but are not limited to species and allelic variants among different RSV subgroups or strains.

The human and bovine strains have the same gene map and encode essentially the same constellation of mRNAs and proteins. For example, each has ORFs encoding NS1, NS2, N, P, M, SH, G, F, M2-1, M2-2 and L proteins. The percent amino acid identity between selected human and bovine proteins is: NS1 (69%), NS2 (84%), N (93%), P (81%), M (89%), SH (38%), G (30%), F (18%), M2-1 (80%), L (77%). In general, therefore, there is clearly discernable correspondence between the genes and genome segments of the two viruses, and the design of substitutions, transfers and insertions is straightforward.

alignment (i.e., resulting in the highest percentage of sequence similarity over the comparison window) generated by the various methods is selected. The term "sequence identity" means that two polynucleotide sequences are identical (i.e., on a nucleotide-by-nucleotide basis) over the window of comparison. The term "percentage of sequence identity" is calculated by comparing two optimally aligned sequences over the window of comparison, determining the number of positions at which the identical nucleic acid base (e.g., A, T, C, G, U, or I) occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison (i.e., the window size), and multiplying the result by 100 to yield the percentage of sequence identity.

Corresponding residue positions between bovine and human RSV may be divergent, identical or may differ by conservative amino acid substitutions. Conservative amino acid substitutions refer to the interchangeability of residues having similar side chains. For example, a conservative group of amino acids having aliphatic side chains is glycine, alanine, valine, leucine, and isoleucine; a group of amino acids having aliphatic-hydroxyl side chains is serine and threonine; a group of amino acids having amide-containing side chains is asparagine and glutamine; a group of amino acids having aromatic side chains is phenylalanine, tyrosine, and tryptophan; a group of amino acids having basic side chains is lysine, arginine, and histidine; and a group of amino acids having sulfur-containing side chains is cysteine and methionine. Preferred conservative amino acids substitution groups are: valine-leucine-isoleucine, phenylalanine-tyrosine, lysine-arginine, alanine-valine, and asparagine-glutamine. Stereoisomers (e.g., D-amino acids) of the twenty conventional amino acids, unnatural amino acids such as α,α -disubstituted amino acids, N-alkyl amino acids, lactic acid, and other unconventional amino acids may also be suitable components for polypeptides of the present invention. Examples of unconventional amino acids include: 4-hydroxyproline, γ -carboxyglutamate, ϵ -N,N,N-trimethyllysine, ϵ -N-acetyllysine, O-phosphoserine, N-acetylserine, N-formylmethionine, 3-methylhistidine, 5-hydroxylysine, ω -N-methylarginine, and other amino and imino acids (e.g., 4-hydroxyproline). Moreover, amino acids may be modified by glycosylation, phosphorylation and the like.

The present invention employs cDNA-based methods to construct a variety of recombinant, human-bovine chimeric RSV viruses and subviral particles. These

recombinant RSV offer improved characteristics of attenuation and immunogenicity for use as vaccine agents. Desired phenotypic changes that are engineered into human-bovine chimeric RSV include, but are not limited to, attenuation in culture or in a selected host environment, resistance to reversion from the attenuated phenotype, enhanced immunogenic characteristics (e.g., as determined by enhancement, or diminution, of an elicited immune response), upregulation or downregulation of transcription and/or translation of selected viral products, etc. In preferred aspects of the invention, attenuated, human-bovine chimeric RSV are produced in which the chimeric genome or antigenome is further modified by introducing one or more attenuating mutations specifying an attenuating phenotype. These mutations may be generated de novo and tested for attenuating effects according to a rational design mutagenesis strategy as described in the above-incorporated references. Alternatively, the attenuating mutations can be identified in a biologically derived mutant RSV and thereafter incorporated into the human-bovine chimeric RSV of the invention.

Attenuating mutations in biologically derived RSV for incorporation within a human-bovine chimeric RSV vaccine strain may occur naturally or may be introduced into wild-type RSV strains by well known mutagenesis procedures. For example, incompletely attenuated parental RSV strains can be produced by chemical mutagenesis during virus growth in cell cultures to which a chemical mutagen has been added, by selection of virus that has been subjected to passage at suboptimal temperatures in order to introduce growth restriction mutations, or by selection of a mutagenized virus that produces small plaques (sp) in cell culture, as generally described herein and in USSN U.S. Patent No. 5,922,326, issued July 13, 1999, incorporated herein by reference.

By "biologically derived RSV" is meant any RSV not produced by recombinant means. Thus, biologically derived RSV include naturally occurring RSV of all subgroups and strains, including, e.g., naturally occurring RSV having a wild-type genomic sequence and RSV having genomic variations from a reference wild-type RSV sequence, e.g., RSV having a mutation specifying an attenuated phenotype. Likewise, biologically derived RSV include RSV mutants derived from a parental RSV strain by, inter alia, artificial mutagenesis and selection procedures.

To produce a satisfactorily attenuated RSV from biologically derived strains, mutations are preferably introduced into a parental strain which has been incompletely or partially attenuated, such as the well known ts-1 or ts-1NG or cpRSV mutants of the A2

strain of RSV subgroup A, or derivatives or subclones thereof. Using these and other partially attenuated strains additional mutation(s) can be generated that further attenuate the strain, e.g., to a desired level of restricted replication in a mammalian host, while retaining sufficient immunogenicity to confer protection in vaccinees.

Partially attenuated mutants of the subgroup A or B virus can be produced by well known methods of biologically cloning wild-type virus in an acceptable cell substrate and developing, e.g., cold-passaged mutants thereof, subjecting the virus to chemical mutagenesis to produce ts mutants, or selecting small plaque or similar phenotypic mutants (see, e.g., Murphy et al., International Publication WO 93/21310, incorporated herein by reference). For virus of subgroup B, an exemplary, partially attenuated parental virus is cp 23, which is a mutant of the B1 strain of subgroup B.

Various known selection techniques may be combined to produce partially attenuated mutants from non-attenuated subgroup A or B strains which are useful for further derivatization as described herein. Further, mutations specifying attenuated phenotypes may be introduced individually or in combination in incompletely attenuated subgroup A or B virus to produce vaccine virus having multiple, defined attenuating mutations that confer a desired level of attenuation and immunogenicity in vaccinees.

As noted above, production of a sufficiently attenuated biologically derived RSV mutant can be accomplished by several known methods. One such procedure involves subjecting a partially attenuated virus to passage in cell culture at progressively lower, attenuating temperatures. For example, whereas wild-type virus is typically cultivated at about 34--37°C, the partially attenuated mutants are produced by passage in cell cultures (e.g., primary bovine kidney cells) at suboptimal temperatures, e.g., 20-26°C. Thus, the cp mutant or other partially attenuated strain, e.g., ts-1 or spRSV, is adapted to efficient growth at a lower temperature by passage in MRC-5 or Vero cells, down to a temperature of about 20-24°C, preferably 20-22°C. This selection of mutant RSV during cold-passage substantially reduces any residual virulence in the derivative strains as compared to the partially attenuated parent.

Alternatively, specific mutations can be introduced into biologically derived RSV by subjecting a partially attenuated parent virus to chemical mutagenesis, e.g., to introduce ts mutations or, in the case of viruses which are already ts, additional ts mutations

be satisfactorily attenuated. Nucleotide sequence analysis of some of these attenuated viruses indicates that each level of increased attenuation is associated with specific nucleotide and amino acid substitutions. The above-incorporated references also disclose how to routinely distinguish between silent incidental mutations and those responsible for phenotype differences by introducing the mutations, separately and in various combinations, into the genome or antigenome of infectious RSV clones. This process coupled with evaluation of phenotype characteristics of parental and derivative virus identifies mutations responsible for such desired characteristics as attenuation, temperature sensitivity, cold-adaptation, small plaque size, host range restriction, etc.

Mutations thus identified are compiled into a "menu" and are then introduced as desired, singly or in combination, to calibrate a human-bovine chimeric RSV vaccine virus to an appropriate level of attenuation, immunogenicity, genetic resistance to reversion from an attenuated phenotype, etc., as desired. Preferably, the chimeric RSV of the invention are attenuated by incorporation of at least one, and more preferably two or more, attenuating mutations identified from such a menu, which may be defined as a group of known mutations within a panel of biologically derived mutant RSV strains. Preferred panels of mutant RSV strains described herein are cold passaged (cp) and/or temperature sensitive (ts) mutants, for example a panel comprised of RSV mutants designated cpts RSV 248 (ATCC VR 2450), cpts RSV 248/404 (ATCC VR 2454), cpts RSV 248/955 (ATCC VR 2453), cpts RSV 530 (ATCC VR 2452), cpts RSV 530/1009 (ATCC VR 2451), cpts RSV 530/1030 (ATCC VR 2455), RSV B-1 cp52/2B5 (ATCC VR 2542), and RSV B-1 cp-23 (ATCC VR 2579) (each deposited under the terms of the Budapest Treaty with the American Type Culture Collection (ATCC) of 10801 University Boulevard, Manassas, Virginia 20110-2209, U.S.A., and granted the above identified accession numbers).

From this exemplary panel of biologically derived mutants, a large menu of attenuating mutations are provided which can each be combined with any other mutation(s) within the panel for calibrating the level of attenuation in a recombinant, human-bovine chimeric RSV for vaccine use. Additional mutations may be derived from RSV having non-ts and non-cp attenuating mutations as identified, e.g., in small plaque (sp), cold-adapted (ca) or host-range restricted (hr) mutant strains. Attenuating mutations may be selected in coding portions of a donor or recipient RSV gene or in non-coding regions such as a cis-regulatory sequence. For example, attenuating mutations may include single or multiple base changes

in a gene start sequence, as exemplified by a single or multiple base substitution in the M2 gene start sequence at nucleotide 7605.

Human-bovine chimeric RSV designed and selected for vaccine use often have at least two and sometimes three or more attenuating mutations to achieve a satisfactory level of attenuation for broad clinical use. In one embodiment, at least one attenuating mutation occurs in the RSV polymerase gene (either in the donor or recipient gene) and involves a nucleotide substitution specifying an amino acid change in the polymerase protein specifying a temperature-sensitive (ts) phenotype. Exemplary human-bovine chimeric RSV in this context incorporate one or more nucleotide substitutions in the large polymerase gene L resulting in an amino acid change at amino acid Asn43, Phe521, Gln831, Met1169, or Tyr1321, as exemplified by the changes, Leu for Phe521, Leu for Gln831, Val for Met1169, and Asn for Tyr1321. Alternately or additionally, chimeric RSV of the invention may incorporate a ts mutation in a different RSV gene, e.g., in the M2 gene. Preferably, two or more nucleotide changes are incorporated in a codon specifying an attenuating mutation, e.g., in a codon specifying a ts mutation, thereby decreasing the likelihood of reversion from an attenuated phenotype.

In accordance with the methods of the invention, human-bovine chimeric RSV can be readily constructed and characterized that incorporate at least one and up to a full complement of attenuating mutations present within a panel of biologically derived mutant RSV strains. Thus, mutations can be assembled in any combination from a selected panel of mutants, for example, cpts RSV 248 (ATCC VR 2450), cpts RSV 248/404 (ATCC VR 2454), cpts RSV 248/955 (ATCC VR 2453), cpts RSV 530 (ATCC VR 2452), cpts RSV 530/1009 (ATCC VR 2451), cpts RSV 530/1030 (ATCC VR 2455), RSV B-1 cp52/2B5 (ATCC VR 2542), and RSV B-1 cp-23 (ATCC VR 2579). In this manner, attenuation of chimeric vaccine candidates can be finely calibrated for use in one or more classes of patients, including seronegative infants.

In more specific embodiments, human-bovine chimeric RSV for vaccine use incorporate at least one and up to a full complement of attenuating mutations specifying a temperature-sensitive and/or attenuating amino acid substitution at Asn43, Phe521, Gln831, Met1169 or Tyr1321 in the RSV polymerase gene L, or a temperature-sensitive nucleotide substitution in the gene-start sequence of gene M2. Alternatively or additionally, chimeric RSV of claim may incorporate at least one and up to a full complement of mutations from

RSV. As used herein, site-specific mutations include insertions, substitutions, deletions or rearrangements of from 1 to 3, up to about 5-15 or more altered nucleotides (e.g., altered from a wild-type RSV sequence, from a sequence of a selected mutant RSV strain, or from a parent recombinant RSV clone subjected to mutagenesis). Such site-specific mutations may be incorporated at, or within the region of, a selected, biologically derived mutation. Alternatively, the mutations can be introduced in various other contexts within an RSV clone, for example at or near a cis-acting regulatory sequence or nucleotide sequence encoding a protein active site, binding site, immunogenic epitope, etc. Site-specific RSV mutants typically retain a desired attenuating phenotype, but may additionally exhibit altered phenotypic characteristics unrelated to attenuation, e.g., enhanced or broadened immunogenicity, and/or improved growth. Further examples of desired, site-specific mutants include recombinant RSV designed to incorporate additional, stabilizing nucleotide mutations in a codon specifying an attenuating mutation. Where possible, two or more nucleotide substitutions are introduced at codons that specify attenuating amino acid changes in a parent mutant or recombinant RSV clone, yielding a biologically derived or recombinant RSV having genetic resistance to reversion from an attenuated phenotype. In other embodiments, site-specific nucleotide substitutions, additions, deletions or rearrangements are introduced upstream (N-terminal direction) or downstream (C-terminal direction), e.g., from 1 to 3, 5-10 and up to 15 nucleotides or more 5' or 3', relative to a targeted nucleotide position, e.g., to construct or ablate an existing cis-acting regulatory element.

In addition to single and multiple point mutations and site-specific mutations, changes to human-bovine chimeric RSV disclosed herein include deletions, insertions, substitutions or rearrangements of whole genes or genome segments. These mutations may alter small numbers of bases (e.g., from 15-30 bases, up to 35-50 bases or more), large blocks of nucleotides (e.g., 50-100, 100-300, 300-500, 500-1,000 bases), or nearly complete or complete genes (e.g., 1,000-1,500 nucleotides, 1,500-2,500 nucleotides, 2,500-5,000, nucleotides, 5,000-6,500 nucleotides or more) in the donor or recipient genome or antigenome, depending upon the nature of the change (i.e., a small number of bases may be changed to insert or ablate an immunogenic epitope or change a small genome segment, whereas large block(s) of bases are involved when genes or large genome segments are added, substituted, deleted or rearranged).

In additional aspects, the invention provides for supplementation of mutations adopted into a chimeric RSV clone from biologically derived RSV, e.g., cp and ts mutations, with additional types of mutations involving the same or different genes in a further modified chimeric RSV clone. RSV encodes ten mRNAs and ten or eleven proteins. Three
5 of these are transmembrane surface proteins, namely the attachment G protein, fusion F protein involved in penetration, and small hydrophobic SH protein. G and F are the major viral neutralization and protective antigens. Four additional proteins are associated with the viral nucleocapsid, namely the RNA binding protein N, the phosphoprotein P, the large polymerase protein L, and the transcription elongation factor M2 ORF1. A second ORF in
10 M2, the M2-2 ORF encodes an important RNA regulatory factor. The matrix M protein is part of the inner virion and probably mediates association between the nucleocapsid and the envelope. Finally, there are two nonstructural proteins, NS1 and NS2, of unknown function. Each of these proteins can be selectively altered in terms of expression levels, or can be added deleted, substituted or rearranged, in whole or in part, alone or in combination with
15 other desired modifications, to yield a human-bovine chimeric RSV exhibiting novel vaccine characteristics.

Thus, in addition to or in combination with attenuating mutations adopted from biologically derived RSV mutants, the present invention also provides a range of additional methods for attenuating or otherwise modifying the phenotype of human-bovine
20 chimeric RSV based on recombinant engineering of infectious RSV clones. A variety of alterations can be produced in an isolated polynucleotide sequence encoding the donor gene or genome segment or the background genome or antigenome for incorporation into infectious clones. More specifically, to achieve desired structural and phenotypic changes in chimeric RSV, the invention allows for introduction of modifications which delete,
25 substitute, introduce, or rearrange a selected nucleotide or plurality of nucleotides from a parent chimeric genome or antigenome, as well as mutations which delete, substitute, introduce or rearrange whole gene(s) or genome segment(s), within a human-bovine chimeric RSV clone.

Desired modifications of infectious human-bovine chimeric RSV are
30 typically selected to specify a desired phenotypic change, e.g., a change in viral growth, temperature sensitivity, ability to elicit a host immune response, attenuation, etc. These changes can be brought about either in a donor or recipient genome or antigenome by, e.g.,

example, ablation by deletion of a non-essential gene such as SH results in enhanced viral growth in culture. Without wishing to be bound by theory, this effect is likely due in part to a reduced nucleotide length of the viral genome. In the case of one exemplary SH-minus clone, the modified viral genome is 14,825 nt long, 398 nucleotides less than wild-type. By engineering similar mutations that decrease genome size, e.g., in other coding or noncoding regions elsewhere in the RSV genome, such as in the P, M, F and M2 genes, the invention provides several readily obtainable methods and materials for improving chimeric RSV growth.

In addition, a variety of other genetic alterations can be produced in a RSV genome or antigenome for incorporation into infectious human-bovine chimeric RSV, alone or together with one or more attenuating mutations adopted from a biologically derived mutant RSV. Additional heterologous genes and genome segments (e.g. from different RSV genes, different RSV strains or types, or non-RSV sources) may be inserted in whole or in part, the order of genes changed, gene overlap removed, an RSV genome promoter replaced with its antigenome counterpart, portions of genes removed or substituted, and even entire genes deleted. Different or additional modifications in the sequence can be made to facilitate manipulations, such as the insertion of unique restriction sites in various intergenic regions or elsewhere. Nontranslated gene sequences can be removed to increase capacity for inserting foreign sequences.

Also provided within the invention are genetic modifications in a human-bovine chimeric RSV which alter or ablate the expression of a selected gene or genome segment without removing the gene or genome segment from the chimeric RSV clone. For example, this can be achieved by introducing a frame shift mutation or termination codon within a selected coding sequence, changing the position of a gene or introducing an upstream start codon to alter its rate of expression, or changing GS and/or GE transcription signals to alter phenotype (e.g., growth, temperature restrictions on transcription, etc.). In one such example, expression of the M2-2 ORF is knocked out, which yields highly desired phenotypic changes for development of a vaccine virus, including altered growth kinetics, reduced RNA replication and increased transcription. Thus, the M2-2 knock-out exhibits significant attenuation with a concomitant increase, rather than decrease, in antigen expression.

four-fold, up to ten-fold or more compared to wild-type levels often attended by a commensurate decrease in expression levels for reciprocally, positionally substituted genes. In one example, the NS2 gene (second in order in the RSV gene map) is substituted in position for the SH gene (sixth in order), yielding a predicted decrease in expression of NS2.

- 5 In other exemplary embodiments, the F and G genes are transpositioned singly or together to a more promoter-proximal or promoter-distal site within the chimeric RSV gene map to achieve higher or lower levels of gene expression, respectively. These and other transpositioning changes yield novel chimeric RSV clones having attenuated phenotypes, for example due to decreased expression of selected viral proteins involved in RNA replication, 10 or having other desirable properties such as increased antigen expression.

- Infectious human-bovine chimeric RSV clones of the invention can also be engineered according to the methods and compositions disclosed herein to enhance immunogenicity and induce a level of protection greater than that provided by infection with a wild-type RSV or a parent chimeric RSV. For example, an immunogenic epitope from a 15 heterologous RSV strain or type, or from a non-RSV source such as PIV, can be added to a chimeric clone by appropriate nucleotide changes in the polynucleotide sequence encoding the chimeric genome or antigenome. Alternatively, chimeric RSV can be engineered to add or ablate (e.g., by amino acid insertion, substitution or deletion) immunogenic proteins, protein domains, or forms of specific proteins (such as the secreted form of G) associated 20 with desirable or undesirable immunological reactions.

- Within the methods of the invention, additional genes or genome segments may be inserted into or proximate to the human-bovine chimeric RSV genome or antigenome. These genes may be under common control with recipient genes, or may be under the control of an independent set of transcription signals. Genes of interest include the 25 RSV genes identified above, as well as non-RSV genes. Non-RSV genes of interest include those encoding cytokines (e.g., IL-2 through IL-18, especially IL-2, IL-6 and IL-12, IL-18, etc.), gamma-interferon, and proteins rich in T helper cell epitopes. These additional proteins can be expressed either as a separate protein, or as a chimera engineered from a second copy of one of the RSV proteins, such as SH. This provides the ability to modify and 30 improve the immune responses against RSV both quantitatively and qualitatively.

In exemplary embodiments of the invention, insertion of foreign genes or genome segments, and in some cases of noncoding nucleotide sequences, within a human-

bovine chimeric RSV genome results in a desired increase in genome length causing yet additional, desired phenotypic effects. Increased genome length results in attenuation of the resultant RSV, dependent in part upon the length of the insert. In addition, the expression of certain proteins, e.g. a cytokine, from a non-RSV gene inserted into human-bovine chimeric RSV will result in attenuation of the virus due to the action of the protein. Exemplary cytokines that yield an infectious, attenuated viral phenotype and high level cytokine expression from RSV transfected cells include interleukin-2 (IL-2), IL-4, GM-CSF, and γ -interferon. Additional effects including augmentation of cellular and or humoral immune responses will also attend introduction of cytokines into human-bovine chimeric RSV of the invention.

Deletions, insertions, substitutions and other mutations involving changes of whole viral genes or genome segments within human-bovine chimeric RSV of the invention yield highly stable vaccine candidates, which are particularly important in the case of immunosuppressed individuals. Many of these changes will result in attenuation of resultant vaccine strains, whereas others will specify different types of desired phenotypic changes. For example, accessory (i.e., not essential for *in vitro* growth) genes are excellent candidates to encode proteins that specifically interfere with host immunity (see, e.g., Kato et al., EMBO J. 16:578-87, 1997, incorporated herein by reference). Ablation of such genes in chimeric vaccine viruses is expected to reduce virulence and pathogenesis and/or improve immunogenicity.

In alternative aspects of the invention, the infectious human-bovine chimeric RSV produced from a cDNA-expressed genome or antigenome can be any of the RSV or RSV-like strains, e.g., human, bovine, murine, etc., or of any pneumovirus, e.g., pneumonia virus of mice avian pneumovirus (previously called turkey rhinotracheitis virus). To engender a protective immune response, the RSV strain may be one which is endogenous to the subject being immunized, such as human RSV being used to immunize humans. The genome or antigenome of endogenous RSV can be modified, however, to express RSV genes or genome segments from a combination of different sources, e.g., a combination of genes or genome segments from different RSV species, subgroups, or strains, or from an RSV and another respiratory pathogen such as PIV.

In certain embodiments of the invention, human-bovine chimeric RSV are provided wherein genes or genome segments within a human or bovine RSV (e.g., a human

RSV background genome or antigenome) are replaced with counterpart heterologous genes or genome segments from a non-human, non-bovine RSV, e.g., a murine RSV.

Substitutions, deletions, and additions of RSV genes or genome segments in this context can include part or all of one or more of the NS1, NS2, N, P, M, SH, M2(ORF1), M2(ORF2) and

5 L genes, or parts of the G and F genes which preferably are outside of the major neutralization and protective epitopes. Also, human or bovine RSV cis-acting sequences, such as promoter or transcription signals, can be replaced with non-human, non-bovine counterpart sequences. Thus, infectious human-bovine chimeric RSV intended for administration to humans can be a human RSV that has been modified to contain genes from
10 a murine RSV in addition to bovine RSV.

Replacement of a human RSV coding sequence (e.g., of NS1, NS2, SH, G, M2-2) or non-coding sequence (e.g., a promoter, gene-end, gene-start, intergenic or other cis-acting element) with a counterpart bovine or murine RSV sequence yields chimeric RSV having a variety of possible attenuating and other phenotypic effects. In particular, host
15 range and other desired effects arise from substituting a bovine or murine RSV gene imported within a human RSV background, wherein the bovine or murine gene does not function efficiently in a human cell, e.g., from incompatibility of the heterologous sequence or protein with a biologically interactive human RSV sequence or protein (i.e., a sequence or protein that ordinarily cooperates with the substituted sequence or protein for viral
20 transcription, translation, assembly, etc.) or, more typically in a host range restriction, with a cellular protein or some other aspect of the cellular milieu which is different between the permissive and less permissive host. In one such embodiment, a chimeric bovine-human RSV incorporates a substitution of the human RSV NP gene or genome segment with a counterpart bovine NP gene or genome segment, which chimera can optionally be
25 constructed to incorporate additional genetic changes, e.g., point mutations or gene deletions. Chimeric human-bovine RSV bearing heterologous genes or cis-acting elements are selected for host range restriction and other desired phenotypes favorable for vaccine use. In exemplary embodiments, bovine RSV sequences are selected for introduction into human RSV based on known aspects of bovine RSV structure and function, as provided in, e.g.,
30 Pastey et al., J. Gen. Virol. 76:193-197, 1993; Pastey et al., Virus Res. 29:195-202, 1993; Zamora et al., J. Gen. Virol. 73:737-741, 1992; Mallipeddi et al., J. Gen. Virol. 74:2001-2004, 1993; Mallipeddi et al., J. Gen. Virol. 73:2441-2444, 1992; and Zamora et al., Virus

Res. 24:115-121, 1992, each incorporated herein by reference, and in accordance with the teachings disclosed herein.

In other embodiments of the invention, mutations of interest for introduction within human-bovine chimeric RSV are modeled after a tissue culture-adapted

5 nonpathogenic strain of pneumonia virus of mice (the murine counterpart of human RSV) which lacks a cytoplasmic tail of the G protein (Randhawa et al., Virology 207:240-245, 1995). Accordingly, in one aspect of the invention the cytoplasmic and/or transmembrane domains of one or more of the human RSV glycoproteins, F, G and SH, are added, deleted, modified, or substituted within a chimeric RSV using a heterologous counterpart sequence
10 (e.g., a sequence from a cytoplasmic, or transmembrane domain of a F, G, or SH protein of murine pneumonia virus) to achieve a desired attenuation. As another example, a nucleotide sequence at or near the cleavage site of the F protein, or the putative attachment domain of the G protein, can be modified by point mutations, site-specific changes, or by alterations involving entire genes or genome segments to achieve novel effects on viral growth in tissue
15 culture and/or infection and pathogenesis.

In more detailed aspects of the invention, human-bovine chimeric RSV are employed as vectors for protective antigens of other pathogens, particularly respiratory tract pathogens such as parainfluenza virus (PIV). For example, chimeric RSV may be engineered which incorporate sequences that encode protective antigens from PIV to
20 produce infectious, attenuated vaccine virus. The cloning of PIV cDNA and other disclosure for implementing these and other more detailed aspects of the invention is provided in United States Patent Application No. 09/083,793 (corresponding to International Publication No. WO 98/53078) and its priority, U.S. provisional application Serial No. 60/047,575; United States Patent Application No. 09/350,821; and United States Patent Application No.
25 09/586,479 and its priority, provisional application Serial No. 60/143,134, each incorporated herein by reference. These disclosures include description of the following plasmids that may be employed to produce infectious PIV viral clones: p3/7(131) (ATCC 97990); p3/7(131)2G (ATCC 97989); and p218(131) (ATCC 97991); each deposited under the terms of the Budapest Treaty with the American Type Culture Collection (ATCC) of 10801
30 University Boulevard, Manassas, Virginia 20110-2209, U.S.A., and granted the above identified accession numbers.

According to this aspect of the invention, a human-bovine chimeric RSV is provided which comprises a chimera of a bovine and human RSV genomic or antigenomic sequence and at least one PIV sequence, for example a polynucleotide containing sequences from both bovine and human RSV, and PIV1, PIV2, PIV3 or bovine PIV. For example, individual genes of RSV may be replaced with counterpart genes from human PIV, such as the F glycoprotein genes of PIV1, PIV2, or PIV3. Alternatively, a selected, heterologous genome segment, such as a cytoplasmic tail, transmembrane domain or ectodomain of substituted for a counterpart genome segment in, e.g., the same gene in RSV, within a different gene in RSV, or into a non-coding sequence of the RSV genome or antigenome. In one embodiment, a genome segment from an F gene of HPIV3 is substituted for a counterpart human RSV genome segment to yield constructs encoding chimeric proteins, e.g. fusion proteins having a cytoplasmic tail and/or transmembrane domain of RSV fused to an ectodomain of RSV to yield a novel attenuated virus, and/or a multivalent vaccine immunogenic against both PIV and RSV. The cloning of PIV cDNA and other disclosure for implementing these and other more detailed aspects of the invention is provided in United States Patent Application No. 09/083,793 (corresponding to International Publication No. WO 98/53078) and its priority, provisional application Serial No. 60/047,575; United States Patent Application No. 09/350,821; and United States Patent Application No. 09/586,479 and its priority, provisional application Serial No. 60/143,134, each incorporated herein by reference.

Thus human-bovine chimeric RSV of the invention may be employed as vectors for expressing antigenic determinants of a wide variety of other pathogens. Typically, a bovine-human chimeric genome or antigenome is employed as partial or complete RSV vector genome or antigenome. Incorporated within the vector genome or antigenome is one or more heterologous genes or genome segments encoding antigenic determinant(s) of one or more heterologous pathogens. In certain embodiments, the heterologous pathogen is a heterologous RSV, and the heterologous gene(s) or genome segment(s) incorporated in the human-bovine chimeric RSV vector genome or antigenome encode(s) one or more RSV NS1, NS2, N, P, M, SH, M2(ORF1), M2(ORF2), L, F or G protein(s) or fragment(s) thereof. For example, the vector genome or antigenome may include a partial or complete RSV A genome or antigenome, and the heterologous gene or genome segment encoding the antigenic determinant may be of a RSV B subgroup virus.

In other alternative embodiments, the human-bovine chimeric RSV is employed as a vector for expressing one or more antigenic determinants of a non-RSV pathogen. The chimeric vector virus thus constructed may express antigenic determinants of RSV as well as of the non-RSV pathogen, or may elicit a mono-specific immune response against the non-RSV pathogen. In one embodiment, human-bovine chimeric RSV incorporate one or more HPIV1, HPIV2, or HPIV3 gene(s) or genome segment(s) encoding one or more HN and/or F glycoprotein(s) or antigenic domain(s), fragment(s) or epitope(s) thereof. In more detailed aspects, a transcription unit comprising an open reading frame (ORF) of an HPIV2 HN or F gene is added to or incorporated within the chimeric HRSV vector genome or antigenome.

Human-bovine chimeric RSV employed as vectors include chimeric RSV wherein the vector genome or antigenome is a partial or complete BRSV genome or antigenome, and the heterologous gene(s) or genome segment(s) encoding the antigenic determinant(s) is/are of one or more HRSV(s). For example, the partial or complete BRSV genome or antigenome may incorporate one or more gene(s) or genome segment(s) encoding one or more HRSV glycoprotein genes selected from F, G and SH, or one or more genome segment(s) encoding cytoplasmic domain, transmembrane domain, ectodomain or immunogenic epitope portion(s) of F, G, and/or SH of HRSV.

In yet additional vector constructs of the invention, the vector genome or antigenome includes a partial or complete HRSV or BRSV genome or antigenome, and the heterologous pathogen is selected from measles virus, subgroup A and subgroup B respiratory syncytial viruses, mumps virus, human papilloma viruses, type 1 and type 2 human immunodeficiency viruses, herpes simplex viruses, cytomegalovirus, rabies virus, Epstein Barr virus, filoviruses, bunyaviruses, flaviviruses, alphaviruses and influenza viruses. From these and other pathogens, useful heterologous antigenic determinant(s) for incorporation within the human-bovine chimeric RSV may be selected from measles virus HA and F proteins, subgroup A or subgroup B respiratory syncytial virus F, G, SH and M2 proteins, mumps virus HN and F proteins, human papilloma virus L1 protein, type 1 or type 2 human immunodeficiency virus gp160 protein, herpes simplex virus and cytomegalovirus gB, gC, gD, gE, gG, gH, gI, gJ, gK, gL, and gM proteins, rabies virus G protein, Epstein Barr Virus gp350 protein; filovirus G protein, bunyavirus G protein, Flavivirus E and NS1 proteins, and alphavirus E protein, and antigenic domains, fragments and epitopes thereof.

antigenome or genome cDNA, or any subfragment thereof, can be used as template for oligonucleotide-directed mutagenesis. This can be through the intermediate of a single-stranded phagemid form, such as using the Muta-gene® kit of Bio-Rad Laboratories (Richmond, CA) or a method using a double-stranded plasmid directly as template such as the Chameleon mutagenesis kit of Stratagene (La Jolla, CA), or by the polymerase chain reaction employing either an oligonucleotide primer or template which contains the mutation(s) of interest. A mutated subfragment can then be assembled into the complete antigenome or genome cDNA. A variety of other mutagenesis techniques are known and available for use in producing the mutations of interest in the RSV antigenome or genome cDNA. Mutations can vary from single nucleotide changes to replacement of large cDNA pieces containing one or more genes or genome regions.

Thus, in one illustrative embodiment mutations are introduced by using the Muta-gene phagemid *in vitro* mutagenesis kit available from Bio-Rad. In brief, cDNA encoding a portion of an RSV genome or antigenome is cloned into the plasmid pTZ18U, and used to transform CJ236 cells (Life Technologies). Phagemid preparations are prepared as recommended by the manufacturer. Oligonucleotides are designed for mutagenesis by introduction of an altered nucleotide at the desired position of the genome or antigenome. The plasmid containing the genetically altered genome or antigenome fragment is then amplified and the mutated piece is then reintroduced into the full-length genome or antigenome clone.

The ability to introduce defined mutations into infectious RSV has many applications, including the analyses of RSV molecular biology and pathogenesis. For example, the functions of RSV proteins can be investigated and manipulated by introducing mutations which ablate or reduce their level of expression, or which yield mutant protein. In one exemplary embodiment, recombinant RSV is constructed in which expression of a viral gene, namely the SH gene, is ablated, e.g., by deletion of the mRNA coding sequence and flanking transcription signals. Surprisingly, not only could this virus be recovered, but it grew efficiently in tissue culture. In fact, its growth was substantially increased over that of the wild-type, based on both yield of infectious virus and on plaque size. This improved growth in tissue culture from the SH deletion and other RSV derivatives of the invention provides useful tools for developing RSV vaccines, which overcome the problem of RSV's poor yield in tissue culture that had complicated production of vaccine virus in other

systems. These deletions are highly stable against genetic reversion, rendering the RSV clones derived therefrom particularly useful as vaccine agents.

In another exemplary embodiment, chimeric RSV are provided that incorporate a mutation that deletes the M2 gene (M2ORF2) (Collins and Wertz, J. Virol. 54:65-71, 1985; Collins et al., J. Gen. Virol. 71:3015-3020, 1990, Collins et al., Proc. Natl. Acad. Sci. USA 93:81-85, 1996, each incorporated herein by reference), or reduces or ablates expression of M2 ORF2 to yield novel RSV vaccine candidates (see U.S. Provisional Patent Application No. 60/143,097, filed by Collins et al. on July 9, 1999, incorporated herein by reference). In preferred aspects, expression of M2 ORF2 is reduced or ablated by modifying the chimeric RSV genome or antigenome to incorporate a frame shift mutation or one or stop codons in M2 ORF2 yielding a "knock out" viral clone. Alternatively, M2 ORF2 is deleted in whole or in part to render the M2-2 protein partially or entirely non-functional or to disrupt its expression altogether to yield a M2 ORF2 "deletion mutant" chimeric RSV. Alternatively, the M2-2 ORF may be transpositioned in the genome or antigenome to a more promoter-proximal or promoter-distal position compared to the natural gene order position of M2-2 gene to up-regulate or down-regulate expression of the M2-2 ORF. In additional embodiments, the M2-2 ORF is incorporated in the genome or antigenome as a separate gene having a gene start and gene end gene end signal, which modification results in up-regulation of the M2-2 ORF.

The chimeric RSV of the invention further modified to incorporate mutations in M2 ORF2 possess highly desirable phenotypic characteristics for vaccine development. The above identified modifications in the chimeric genome or antigenome specify one or more desired phenotypic changes in the resulting virus or subviral particle. Vaccine candidates are thus generated that exhibit one or more characteristics identified as (i) a change in mRNA transcription, (ii) a change in the level of viral protein expression; (iii) a change in genomic or antigenomic RNA replication, (iv) a change in viral growth characteristics, (v), a change in viral plaque size, and/or (vi) a change in cytopathogenicity.

In exemplary RSV chimeras incorporating an M2 ORF 2 deletion or knock out mutation, desired phenotypic changes include attenuation of viral growth compared to growth of a corresponding wild-type or mutant parental RSV strain. In more detailed aspects, viral growth in cell culture may be attenuated by approximately 10-fold or more

attributable to mutations in M2 ORF2. Kinetics of viral growth are also shown to be modified in a manner that is beneficial for vaccine development.

Also included within the invention are chimeric, M2-ORF 2 deletion and knock out mutant RSV that exhibit delayed kinetics of viral mRNA synthesis compared to kinetics of mRNA synthesis of corresponding wild-type or mutant parental RSV strains. Despite these delayed transcription kinetics, these novel vaccine candidates exhibit an increase in cumulative mRNA synthesis compared to parental virus. These phenotypic changes typically are associated with an increase in viral protein accumulation in infected cells compared to protein accumulation in cells infected with wild-type or other parental RSV strains. At the same time, viral RNA replication is reduced in M2 ORF2 chimeric RSV compared to that of a parental RSV strain having normal M2 ORF2 function, whereby accumulation of genomic or antigenomic RNA is reduced.

Within preferred aspects of the invention, chimeric M2 ORF2 deletion and "knock out" RSV are engineered to express undiminished or, more typically, increased levels of viral antigen(s) while also exhibiting an attenuated phenotype. Immunogenic potential is thus preserved due to the undiminished or increased mRNA transcription and antigen expression, while attenuation is achieved through incorporation of the heterologous gene(s) or gene segment(s) and concomitant reductions in RNA replication and virus growth attributable to the M2-ORF 2 deletion and knock out mutation. This novel suite of phenotypic traits is highly desired for vaccine development. Other useful phenotypic changes that are observed in M2 ORF2 deletion and knock out chimeric RSV include a large plaque phenotype and altered cytopathogenicity compared to corresponding wild-type or mutant parental RSV strains.

The invention also provides methods for producing an infectious human-bovine chimeric RSV from one or more isolated polynucleotides, e.g., one or more cDNAs. According to the present invention cDNA encoding a RSV genome or antigenome is constructed for intracellular or *in vitro* coexpression with the necessary viral proteins to form infectious RSV. By "RSV antigenome" is meant an isolated positive-sense polynucleotide molecule which serves as the template for the synthesis of progeny RSV genome. Preferably a cDNA is constructed which is a positive-sense version of the RSV genome, corresponding to the replicative intermediate RNA, or antigenome, so as to minimize the possibility of hybridizing with positive-sense transcripts of the complementing sequences that encode

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An RSV antigenome may be constructed for use in the present invention by assembling cloned cDNA segments, representing in aggregate the complete antigenome, by polymerase chain reaction (PCR; described in, e.g., U.S. Patent Nos. 4,683,195 and 4,683,202, and PCR Protocols: A Guide to Methods and Applications, Innis et al., eds., Academic Press, San Diego, 1990, incorporated herein by reference) of reverse-transcribed copies of RSV mRNA or genome RNA. For example, cDNAs containing the lefthand end of the antigenome, spanning from an appropriate promoter (e.g., T7 RNA polymerase promoter) and the leader region complement to the SH gene, are assembled in an appropriate expression vector, such as a plasmid (e.g., pBR322) or various available cosmid, phage, or DNA virus vectors. The vector may be modified by mutagenesis and/or insertion of synthetic polylinker containing unique restriction sites designed to facilitate assembly. For example, a plasmid vector described herein was derived from pBR322 by replacement of the PstI-EcoRI fragment with a synthetic DNA containing convenient restriction enzyme sites. Use of pBR322 as a vector stabilized nucleotides 3716-3732 of the RSV sequence, which otherwise sustained nucleotide deletions or insertions, and propagation of the plasmid was in bacterial strain DH10B to avoid an artifactual duplication and insertion which otherwise occurred in the vicinity of nt 4499. For ease of preparation the G, F and M2 genes can be assembled in a separate vector, as can be the L and trailer sequences. The right-hand end (e.g., L and trailer sequences) of the antigenome plasmid may contain additional sequences as desired, such as a flanking ribozyme and tandem T7 transcriptional terminators. The ribozyme can be hammerhead type (e.g., Grosfeld et al., J. Virol. 69:5677-5686, 1995), which would yield a 3' end containing a single nonviral nucleotide, or can any of the other suitable ribozymes such as that of hepatitis delta virus (Perrotta et al., Nature 350:434-436, 1991) which would yield a 3' end free of non-RSV nucleotides. A middle segment (e.g., G-to-M2 piece) is inserted into an appropriate restriction site of the leader-to-SH plasmid, which in turn is the recipient for the L-trailer-ribozyme-terminator piece, yielding a complete antigenome. In an illustrative example described herein, the leader end was constructed to abut the promoter for T7 RNA polymerase which included three transcribed G residues for optimal activity; transcription donates these three nonviral G's to the 5' end of the antigenome. These three nonviral G residues can be omitted to yield a 5' end free of nonviral nucleotides. To generate a nearly correct 3' end, the trailer end was constructed to be adjacent to a hammerhead ribozyme, which upon cleavage would donate a single 3'-phosphorylated U residue to the 3' end of the encoded RNA.

In certain embodiments of the invention, complementing sequences encoding proteins necessary to generate a transcribing, replicating RSV nucleocapsid are provided by one or more helper viruses. Such helper viruses can be wild-type or mutant. Preferably, the helper virus can be distinguished phenotypically from the virus encoded by the RSV cDNA.

5 For example, it is desirable to provide monoclonal antibodies which react immunologically with the helper virus but not the virus encoded by the RSV cDNA. Such antibodies can be neutralizing antibodies. In some embodiments, the antibodies can be used to neutralize the helper virus background to facilitate identification and recovery of the recombinant virus, or in affinity chromatography to separate the helper virus from the recombinant virus.

10 Mutations can be introduced into the RSV cDNA which render the recombinant RSV nonreactive or resistant to neutralization with such antibodies.

A variety of nucleotide insertions and deletions can be made in the human-bovine chimeric RSV genome or antigenome to generate a properly attenuated clone. The nucleotide length of the genome of wild-type human RSV (15,222 nucleotides) is a multiple
15 of six, and members of the Paramyxovirus and Morbillivirus genera typically abide by a "rule of six," i.e., genomes (or minigenomes) replicate efficiently only when their nucleotide length is a multiple of six (thought to be a requirement for precise spacing of nucleotide residues relative to encapsidating NP protein). Alteration of RSV genome length by single residue increments had no effect on the efficiency of replication, and sequence analysis of
20 several different minigenome mutants following passage showed that the length differences were maintained without compensatory changes. Thus, RSV lacks the strict requirement of genome length being a multiple of six, and nucleotide insertions and deletions can be made in the RSV genome or antigenome without defeating replication of the recombinant RSV of the present invention.

25 Alternative means to construct cDNA encoding a human-bovine chimeric RSV genome or antigenome include by reverse transcription-PCR using improved PCR conditions (e.g., as described in Cheng et al., Proc. Natl. Acad. Sci. USA 91:5695-5699, 1994; Samal et al., J. Virol. 70:5075-5082, 1996, each incorporated herein by reference) to reduce the number of subunit cDNA components to as few as one or two pieces. In other
30 embodiments different promoters can be used (e.g., T3, SP6) or different ribozymes (e.g., that of hepatitis delta virus. Different DNA vectors (e.g., cosmids) can be used for propagation to better accommodate the large size genome or antigenome.

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The N, P and L proteins, necessary for RNA replication, require an RNA polymerase elongation factor such as the M2(ORF1) protein for processive transcription. Thus M2(ORF1) or a substantially equivalent transcription elongation factor for negative strand RNA viruses is required for the production of infectious RSV and is a necessary component of functional nucleocapsids during productive infection.

The need for the M2(ORF1) protein is consistent with its role as a transcription elongation factor. The need for expression of the RNA polymerase elongation factor protein for negative strand RNA viruses is a feature of the present invention. M2(ORF1) can be supplied by expression of the complete M2-gene, either by the chimeric genome or antigenome or by coexpression therewith, although in this form the second ORF2 may also be expressed and have an inhibitory effect on RNA replication. Therefore, for production of infectious virus using the complete M2 gene the activities of the two ORFs should be balanced to permit sufficient expression of M(ORF1) to provide transcription elongation activity yet not so much of M(ORF2) to inhibit RNA replication. Alternatively, the ORF1 protein is provided from a cDNA engineered to lack ORF2 or which encodes a defective ORF2. Efficiency of virus production may also be improved by co-expression of additional viral protein genes, such as those encoding envelope constituents (i.e., SH, M, G, F proteins).

Isolated polynucleotides (e.g., cDNA) or expressed from the antigenome or genome cDNA, encoding the human-bovine chimeric RSV genome or antigenome and; separately, or in cis, or expressed from the antigenome or genome cDNA, the N, P, L and M2(ORF1) proteins, are inserted by transfection, electroporation, mechanical insertion, transduction or the like, into cells which are capable of supporting a productive RSV infection, e.g., HEp-2, FRhL-DBS2, MRC, and Vero cells. Transfection of isolated polynucleotide sequences may be introduced into cultured cells by, for example, calcium phosphate-mediated transfection (Wigler et al., Cell 14:725, 1978; Corsaro and Pearson, Somatic Cell Genetics 7:603, 1981; Graham and Van der Eb, Virology 52:456, 1973), electroporation (Neumann et al., EMBO J. 1:841-845, 1982), DEAE-dextran mediated transfection (Ausubel et al., (ed.) Current Protocols in Molecular Biology, John Wiley and Sons, Inc., NY, 1987, cationic lipid-mediated transfection (Hawley-Nelson et al., Focus 15:73-79, 1993) or a commercially available transfection reagent, e.g., LipofectACE® (Life Technologies) (each of the foregoing references are incorporated herein by reference).

following replication *in vivo* has been common for ts viruses (Murphy et al., Infect. Immun. 37:235-242, 1982).

To propagate a human-bovine chimeric RSV virus for vaccine use and other purposes, a number of cell lines which allow for RSV growth may be used. RSV grows in a variety of human and animal cells. Preferred cell lines for propagating attenuated RS virus for vaccine use include DBS-FR_hL-2, MRC-5, and Vero cells. Highest virus yields are usually achieved with epithelial cell lines such as Vero cells. Cells are typically inoculated with virus at a multiplicity of infection ranging from about 0.001 to 1.0 or more, and are cultivated under conditions permissive for replication of the virus, e.g., at about 30-37°C and for about 3-5 days, or as long as necessary for virus to reach an adequate titer. Virus is removed from cell culture and separated from cellular components, typically by well known clarification procedures, e.g., centrifugation, and may be further purified as desired using procedures well known to those skilled in the art.

Human-bovine chimeric RSV which has been attenuated and otherwise modified as described herein can be tested in various well known and generally accepted *in vitro* and *in vivo* models to confirm adequate attenuation, resistance to phenotypic reversion, and immunogenicity for vaccine use. In *in vitro* assays, the modified virus (e.g., a multiply attenuated, biologically derived or recombinant RSV) is tested for temperature sensitivity of virus replication, i.e. ts phenotype, and for the small plaque phenotype. Modified viruses are further tested in animal models of RSV infection. A variety of animal models have been described and are summarized in (Meignier et al., eds., Animal Models of Respiratory Syncytial Virus Infection, Merieux Foundation Publication, 1991, which is incorporated herein by reference). A cotton rat model of RSV infection is described in (U.S. 4,800,078 and Prince et al., Virus Res. 3:193-206, 1985), which are incorporated herein by reference, and is considered predictive of attenuation and efficacy in humans and non-human primates. In addition, a primate model of RSV infection using the chimpanzee is predictive of attenuation and efficacy in humans, as is described in detail in (Richardson et al., J. Med. Virol. 3:91-100, 1978; Wright et al., Infect. Immun. 37:397-400, 1982; Crowe et al., Vaccine 11:1395-1404, 1993, each incorporated herein by reference).

RSV model systems, including rodents and chimpanzees for evaluating attenuation and infectivity of RSV vaccine candidates are widely accepted in the art and the data obtained therefrom correlate well with RSV infection and attenuation. The mouse and

cotton rat models are especially useful in those instances in which candidate RSV viruses display inadequate growth in chimpanzees, for example in the case of RSV subgroup B viruses.

In accordance with the foregoing description and based on the examples below, the invention also provides isolated, infectious human-bovine chimeric RSV compositions for vaccine use. The attenuated chimeric virus which is a component of a vaccine is in an isolated and typically purified form. By isolated is meant to refer to RSV which is in other than a native environment of a wild-type virus, such as the nasopharynx of an infected individual. More generally, isolated is meant to include the attenuated virus as a component of a cell culture or other artificial medium where it can be propagated and characterized in a controlled setting. For example, attenuated RSV of the invention may be produced by an infected cell culture, separated from the cell culture and added to a stabilizer.

Chimeric RSV vaccines of the invention contain as an active ingredient an immunogenically effective amount of RSV produced as described herein. Biologically derived or recombinant RSV can be used directly in vaccine formulations, or lyophilized. Lyophilized virus will typically be maintained at about 4°C. When ready for use the lyophilized virus is reconstituted in a stabilizing solution, e.g., saline or comprising SPG, Mg++ and HEPES, with or without adjuvant, as further described below. The biologically derived or recombinantly modified virus may be introduced into a host with a physiologically acceptable carrier and/or adjuvant. Useful carriers are well known in the art, and include, e.g., water, buffered water, 0.4% saline, 0.3% glycine, hyaluronic acid and the like. The resulting aqueous solutions may be packaged for use as is, or lyophilized, the lyophilized preparation being combined with a sterile solution prior to administration, as mentioned above. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents, wetting agents and the like, for example, sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride, sorbitan monolaurate, triethanolamine oleate, and the like. Acceptable adjuvants include incomplete Freund's adjuvant, aluminum phosphate, aluminum hydroxide, or alum, which are materials well known in the art. Preferred adjuvants also include Stimulon® QS-21 (Aquila Biopharmaceuticals, Inc., Farmingham, MA), MPL® (3-O-deacylated monophosphoryl lipid

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a volume of 0.5 ml of a physiologically acceptable diluent or carrier. This has the advantage of simplicity and safety compared to parenteral immunization with a non-replicating vaccine. It also provides direct stimulation of local respiratory tract immunity, which plays a major role in resistance to RSV. Further, this mode of vaccination effectively bypasses the
5 immunosuppressive effects of RSV-specific maternally-derived serum antibodies, which typically are found in the very young. Also, while the parenteral administration of RSV antigens can sometimes be associated with immunopathologic complications, this has never been observed with a live virus.

In all subjects, the precise amount of human-bovine chimeric RSV vaccine
10 administered and the timing and repetition of administration will be determined based on the patient's state of health and weight, the mode of administration, the nature of the formulation, etc. Dosages will generally range from about 10^3 to about 10^6 plaque forming units (PFU) or more of virus per patient, more commonly from about 10^4 to 10^5 PFU virus per patient. In any event, the vaccine formulations should provide a quantity of attenuated
15 RSV of the invention sufficient to effectively stimulate or induce an anti-RSV immune response, e.g., as can be determined by complement fixation, plaque neutralization, and/or enzyme-linked immunosorbent assay, among other methods. In this regard, individuals are also monitored for signs and symptoms of upper respiratory illness. As with administration to chimpanzees, the attenuated virus of the vaccine grows in the nasopharynx of vaccinees at
20 levels approximately 10-fold or more lower than wild-type virus, or approximately 10-fold or more lower when compared to levels of incompletely attenuated RSV.

In neonates and infants, multiple administration may be required to elicit sufficient levels of immunity. Administration should begin within the first month of life, and at intervals throughout childhood, such as at two months, six months, one year and two
25 years, as necessary to maintain sufficient levels of protection against native (wild-type) RSV infection. Similarly, adults who are particularly susceptible to repeated or serious RSV infection, such as, for example, health care workers, day care workers, family members of young children, the elderly, individuals with compromised cardiopulmonary function, may require multiple immunizations to establish and/or maintain protective immune responses.
30 Levels of induced immunity can be monitored by measuring amounts of neutralizing secretory and serum antibodies, and dosages adjusted or vaccinations repeated as necessary to maintain desired levels of protection. Further, different vaccine viruses may be indicated

for administration to different recipient groups. For example, an engineered chimeric RSV strain expressing a cytokine or an additional protein rich in T cell epitopes may be particularly advantageous for adults rather than for infants. RSV vaccines produced in accordance with the present invention can be combined with viruses expressing antigens of another subgroup or strain of RSV to achieve protection against multiple RSV subgroups or strains. Alternatively, the vaccine virus may incorporate protective epitopes of multiple RSV strains or subgroups engineered into one RSV clone as described herein.

Typically when different vaccine viruses are used they will be administered in an admixture simultaneously, but they may also be administered separately. For example, as the F glycoproteins of the two RSV subgroups differ by only about 10% in amino acid sequence, this similarity is the basis for a cross-protective immune response as observed in animals immunized with RSV or F antigen and challenged with a heterologous strain. Thus, immunization with one strain may protect against different strains of the same or different subgroup. However, optimal protection probably will require immunization against both subgroups.

The human-bovine chimeric RSV vaccines of the invention elicit production of an immune response that is protective against serious lower respiratory tract disease, such as pneumonia and bronchiolitis when the individual is subsequently infected with wild--type RSV. While the naturally circulating virus is still capable of causing infection, particularly in the upper respiratory tract, there is a very greatly reduced possibility of rhinitis as a result of the vaccination and possible boosting of resistance by subsequent infection by wild-type virus. Following vaccination, there are detectable levels of host engendered serum and secretory antibodies which are capable of neutralizing homologous (of the same subgroup) wild-type virus *in vitro* and *in vivo*. In many instances the host antibodies will also neutralize wild-type virus of a different, non-vaccine subgroup.

Preferred human-bovine chimeric RSV of the present invention exhibit a very substantial diminution of virulence when compared to wild-type virus that is circulating naturally in humans. The chimeric virus is sufficiently attenuated so that symptoms of infection will not occur in most immunized individuals. In some instances the attenuated virus may still be capable of dissemination to unvaccinated individuals. However, its virulence is sufficiently abrogated such that severe lower respiratory tract infections in the vaccinated or incidental host do not occur.

The level of attenuation of human-bovine chimeric RSV vaccine virus may be determined by, for example, quantifying the amount of virus present in the respiratory tract of an immunized host and comparing the amount to that produced by wild-type RSV or other attenuated RSV which have been evaluated as candidate vaccine strains. For example, the
5 attenuated chimeric virus of the invention will have a greater degree of restriction of replication in the upper respiratory tract of a highly susceptible host, such as a chimpanzee, compared to the levels of replication of wild-type virus, e.g., 10- to 1000-fold less. Also, the level of replication of the attenuated RSV vaccine strain in the upper respiratory tract of the chimpanzee should be less than that of the RSV A2 ts-1 mutant, which was demonstrated
10 previously to be incompletely attenuated in seronegative human infants. In order to further reduce the development of rhinorrhea, which is associated with the replication of virus in the upper respiratory tract, an ideal vaccine candidate virus should exhibit a restricted level of replication in both the upper and lower respiratory tract. However, the attenuated viruses of the invention must be sufficiently infectious and immunogenic in humans to confer
15 protection in vaccinated individuals. Methods for determining levels of RS virus in the nasopharynx of an infected host are well known in the literature. Specimens are obtained by aspiration or washing out of nasopharyngeal secretions and virus quantified in tissue culture or other by laboratory procedure. See, for example, (Belshe et al., J. Med. Virology 1:157-162, 1977; Friedewald et al., J. Amer. Med. Assoc. 204:690-694, 1968; Gharpure et al., J.
20 Virology 3:414-421, 1969; and Wright et al., Arch. Ges. Virusforsch. 41:238-247, 1973), each incorporated herein by reference. The virus can conveniently be measured in the nasopharynx of host animals, such as chimpanzees.

In some instances it may be desirable to combine the human-bovine chimeric RSV vaccines of the invention with vaccines which induce protective responses to other
25 agents, particularly other childhood viruses. For example, a chimeric RSV vaccine of the present invention can be administered simultaneously with parainfluenza virus vaccine, such as described in Clements et al., J. Clin. Microbiol. 29:1175-1182, 1991), which is incorporated herein by reference. In another aspect of the invention the chimeric RSV can be employed as a vector for protective antigens of other respiratory tract pathogens, such as
30 PIV, by incorporating the sequences encoding those protective antigens into the chimeric RSV genome or antigenome which is used to produce infectious chimeric RSV, as described herein.

Infectious rBRSV can be recovered from cloned cDNAs encoding a viral antigenomic RNA and the N, P, M2-1 and L support proteins (Buchholz et al., J. Virol. 73:251-259, 1999), using a strategy similar to that described for HRSV (Collins et al., Proc. Natl. Acad. Sci. USA 92:11563-11567, 1995; Bermingham and Collins, Proc. Natl. Acad. Sci. USA 96:11259-11264, 1999; Bukreyev et al., J. Virol. 70:6634-6641, 1996; Bukreyev et al., J. Virol. 71:8973-8982, 1997; Whitehead et al., J. Virol. 72:4467-4471, 1998; Whitehead et al., Virology 247:232-239, 1998; Collins et al., Virology 259:251-255, 1999; Bukreyev et al., Proc. Natl. Acad. Sci. USA 96:2367-2372, 1999; Juhasz et al., Vaccine 17:1416-1424, 1999; Juhasz et al., J. Virol. 73:5176-5180, 1999; Teng and Collins, J. Virol. 73:466-473, 1999; Whitehead et al., J. Virol. 73:9773-9780, 1999; Whitehead et al., J. Virol. 73:871-877, 1999; Whitehead et al., J. Virol. 73:3438-3442, 1999; Murphy et al., United States Patent No. 5,993,824; each incorporated herein by reference). However, T7 RNA polymerase was supplied through the use of a cell line that stably expresses this enzyme, rather than by infection with a vaccinia virus recombinant expressing the enzyme (Buchholz et al., J. Virol. 73:251-259, 1999).

The cDNA for rBRSV is designed to encode a complete rBRSV antigenomic RNA with authentic 3' and 5' ends without additional heterologous terminal nucleotides. This cDNA encodes infectious rBRSV with *in vitro* growth properties that are essentially indistinguishable from those of its biological parent, the ATue51908 strain (*id.*), which is in turn derived from the A51908 strain supplied by the American Type Culture Collection.

For use within the instant invention, the foregoing plasmid was modified by site-directed mutagenesis according to standard procedures (Kunkel, et al., Methods Enzymol. 154:367-382, 1987; incorporated herein by reference) as follows: First, the unique synthetic NotI site in the NS1 noncoding region (Buchholz, et al., J. Virol. 73:251-9, 1999, incorporated herein by reference) was removed to yield the ATue51908 wild type sequence (GenBank Accession no. AF 092942). Second, synthetic marker restriction sites were introduced into the SH/G (SalI, ATue51908 nt 4,673), the G/F

SphI, ATue51908 nt 5,539) and the F/M2 (*Xho*I, ATue51908 nt 7,471 and *Cla*I, ATue51908 nt 7,485) intergenic regions (Fig. 1B). The resulting plasmid was termed pBRSV18 (the Genbank accession number for the BRSV cDNA in pBRSV18 is AF092942). This cDNA served as the parent for constructing BRSV/HRSV chimeras. The rBRSV recovered from this parental cDNA was essentially indistinguishable from the biologically-

derived ATue51908 virus in terms of *in vitro* growth characteristics, and thus is considered to exhibit a wild type growth phenotype.

The G and F genes (nt 4,674 to 7,551) of the previously-reported recombinant version of HRSV strain A2 (U.S. Provisional Patent Application No. 60/007,083, filed September 27, 1995; U.S. Patent Application No. 08/720,132, filed September 27, 1996; U.S. Provisional Patent Application No. 60/021,773, filed July 15, 1996; U.S. Provisional Patent Application No. 60/046,141, filed May 9, 1997; U.S. Provisional Patent Application No. 60/047,634, filed May 23, 1997; U.S. Patent No. 5,993,824, issued November 30, 1999; International Publication No. WO 98/02530; Collins, et al., Proc. Nat. Acad. Sci. USA 92:11563-11567, 1995; each incorporated herein by reference) were amplified with oligonucleotide primers which introduced a *Sall* site immediately upstream of the G gene and an *XhoI* site immediately downstream of the F gene (Fig. 1B). The PCR product was cloned and the sequence of the insert was confirmed in its entirety. Subsequently, these *Sall* and *XhoI* restriction sites were used to transfer an approximately 2,885 bp fragment spanning the HRSV G and F genes, including gene start and gene end/polyadenylation signals, into pBRSV18, replacing the respective BRSV genes. The resulting plasmid encoding a chimeric BRSV antigenome with the glycoprotein genes derived from HRSV A2 was termed pBRSV/A2-10. Fig. 1A represents a schematic overview of the genome organization of the recombinant viruses (rBRSV and rBRSV/A2) expressed from pBRSV18 and pBRSV/A2-10. The chimeric antigenome synthesized from the plasmid is 15,227 nt in length, 87 nt longer than that of the parental rBRSV.

Recombinant RSVs were recovered from cDNA essentially as described elsewhere herein and in the above-incorporated references. 32-mm dishes of subconfluent BSR T7/5 cells stably expressing phage T7 RNA polymerase were transfected with of 5 µg of the respective full length plasmids (pBRSV18 or pBRSV/A2-10) and a set of four support plasmids (2.5 µg pN, 2.5 µg pP, 0.5 µg, pL, and 0.5 µg pM2) from which the BRSV N, P, L, and M2 proteins are expressed. All cDNA constructs were cloned under control of a T7 promoter. The expression plasmids contained an EMCV IRES sequence, allowing cap-independent translation. Transfections were done using Superfect (Qiagen, Valencia, California) transfection reagent. Two hours after transfection, the supernatant was removed, cells were washed and maintained in minimum essential medium supplemented with 3% fetal calf serum (FCS). Three days after transfection, the cells were split in a 1 to 3 ratio.

Cells and supernatant were harvested 7 days after transfection. Duplicate transfections were overlaid with methylcellulose, incubated 5 days without splitting, and examined by immunochemical staining. This showed that pBRSV18 and pBRSV/A2-10 yielded similar levels of recovered virus, approximately 100 foci per 32-mm dish. By this criterion, the
5 rBRSV/A2 virus exhibited essentially the same viability as the rBRSV parent.

To verify the identities of the recovered viruses, viral RNA was recovered from infected cells and analyzed by reverse transcription (RT)PCR. The reactions were performed with primers designed to copy and amplify the three regions of genomic RNA which contained the marker restriction sites shown in Fig. 1B which would distinguish
10 between ATue51908, rBRSV, and rBRSV/A2. The primers used for RT were antigenome sense, complementary to a region of the BRSV (strain ATue51908) M gene (ATue51908 nt 3612 to 3635), to the BRSV G gene (ATue51908 nt 5372 to 5392) or to the HRSV A2 G gene (HRSV A2 nt 5442-5463), and to the BRSV F gene (ATue51908 nt 7218 to 7240) or HRSV A2 F gene (A2 nt 7309 to 7329). An aliquot of the RT product was used for PCR,
15 using the respective first-strand primer noted above and reverse primers, corresponding to the genomic sequences, downstream of the respective intergenic regions (ATue51908 nt 4886 to 4862 and HRSV A2 4878 to 4856, reverse primers downstream of the SH/G intergenic region; ATue51908 nt 5964 to 5941 and HRSV A2 nt 6055 to 6033, reverse primers downstream of the G/F intergenic region, and ATue51908 nt 7852 to 7832, reverse
20 primer downstream of the F/M2 intergenic region).

As shown in Figs 2A and 2B, the RT-PCR reactions each yielded a single cDNA product whose electrophoretic mobility in each case was consistent with calculated predictions. No PCR product was detected if the RT step was omitted, demonstrating that the product was derived from RNA rather than from contaminating DNA. The RT-PCR
25 products were subjected to digestion with diagnostic restriction enzymes (SalI, StuI, SphI, and XhoI) and analyzed by electrophoresis on an agarose gel. In all cases, the expected restriction pattern was obtained, indicating that each virus contained the expected restriction site markers. Specifically, the ATue51908 biologically-derived virus lacked all four sites within the examined region, both recombinants contained a single SalI located in the SH/G
30 intergenic region and a single XhoI site within the F/M2 region, rBRSV contained a single SphI site within the G/F intergenic region, and rBRSV/A2 contained a single StuI site within this latter region. In addition, the RT-PCR products were sequenced using an automated

sequencer (LI-COR, MWG), which confirmed the expected sequences. Thus, the chimeric rBRSV/A2 virus had the expected structure, and there were no sequence changes during recovery.

5

EXAMPLE II

In vitro Analysis of Recombinant Chimeric rBRSV/A2 Virus

To prepare stocks of virus for further analysis, monolayers of bovine MDBK cells were infected with a multiplicity of infection (MOI) of 0.1 with rBRSV or rBRSV/A2. After 90 minutes of adsorption, the inoculum was removed and cells were maintained in minimum essential medium supplemented with 3% fetal bovine serum (FBS). When the cytopathic effect (CPE) was pronounced, typically after 4 to 7 days of incubation, the medium was adjusted to 100 mM MgSO₄ and to 50 mM HEPES (pH 7.5) in order to stabilize the virus (Fernie and Gerin, Virology 106:141-4, 1980, incorporated herein by reference), and cells and medium were subjected to three rounds of rapid freezing to -70°C and rapid thawing in order to release virus. Cell debris was removed by centrifugation. Biologically-derived HRSV strain Long was propagated in HEp-2 cells in the same manner. Strain Long is very similar to strain A2 with regard to sequence analysis and reactivity with most monoclonal antibodies, and thus serves as a surrogate for strain A2 in these particular assays (Johnson, et al., Proc. Nat. Acad. Sci. USA 84:5625-9, 1987; Johnson and Collins, J. Gen. Virol. 70:1539-47, 1989; Lopez, et al., Virus Res. 10:249-61, 1988, each incorporated herein by reference). The ATue51908 biologically-derived parent was not analyzed in parallel because its *in vitro* growth properties are substantially the same as its recombinant derivative rBRSV, which therefore was used as the BRSV control. Virus titrations were carried out as described previously (Buchholz, et al., J. Virol. 73:251-9, 1999, incorporated herein by reference).

The expression of G protein in human HEp-2 cells by rBRSV, rBRSV/A2 and HRSV strain Long was examined by indirect immunofluorescence. HEp-2 cells were infected with an MOI of 0.1 and, 36 h post infection, were fixed with 80% acetone and incubated at 37°C for 30 minutes with mouse monoclonal antibody (mab) G66 directed to BRSV G (1:1000 dilution), (Furze, et al., J. Gen. Virol. 75:363-70, 1994; Langedijk, et al., J. Virol. 71:4055-61, 1997, each incorporated herein by reference), or mab 021/01G directed

against HRSV G (1:40 dilution), (Garcia-Barreno, et al., *J. Virol.* 63:925-32, 1989; Martínez and Melero, *J. Gen. Virol.* 79:2215-20, 1998, each incorporated herein by reference). Cells were stained with a fluorescein isothiocyanate conjugated goat anti-mouse IgG (Dianova, Hamburg) and counterstained with 0.01% Evans Blue.

As expected, the BRSV-specific mab reacted with rBRSV but not with rBRSV/A2 or HRSV (Table 1). In contrast, the HRSV-specific mab reacted with rBRSV/A2 and HRSV, but not with rBRSV. The patterns of reactivity of rBRSV and HRSV were consistent with their bovine and human specificities, respectively, and the pattern of reactivity of rBRSV/A2 confirmed that it expressed a G protein of human rather than bovine origin. On the other hand, the chimeric rBRSV/A2 virus induced cytopathic effect (CPE) in Hep-2 cells which was more similar to that of rBRSV than HRSV. For example, in cultures infected with HRSV, nearly 100% of the cells showed positive immunofluorescence, compared to 20% infected with rBRSV and 20-30% infected with rBRSV/A2. In addition, HRSV induced pronounced CPE with the formation of large syncytia, whereas rBRSV and rBRSV/A2 caused a reduced level of CPE and the formation of smaller syncytia. Thus, the infectivity and CPE of the chimeric rBRSV/A2 virus in this human cell line more closely resembles that of its rBRSV parent. This indicates that transfer of the surface glycoproteins G and F alone to rBRSV does not confer full permissivity of HRSV for growth in human cells—suggesting that additional HRSV genes help determine growth characteristics in Hep-2 cells.

Table 1. Confirmation by reactivity with monoclonal antibodies^a of the presence of the appropriate F and G glycoproteins in the rBRSV/A2 and rBRSV/LongF recombinant viruses.

mab	specificity	rBRSV ^a	rBRSV/A2 ^a	rBRSV/LongF ^a	HRSV ^a
G66	BRSV G	+	-	+	-
021/1G	HRSV G	-	+	-	+
2F	RSV F	+	+	+	+
44F	HRSV F	-	+	+	+

a. Reactivity in indirect immunofluorescence assay and immunoelectron microscopy.

Multicycle growth of rBRSV, rBRSV/A2, and HRSV strain Long was compared in MDBK and HEp-2 cells (Fig. 3). Equal numbers of subconfluent MDBK cells and HEp-2 cells in 24-well dishes were infected with virus at an MOI of 0.1. After 90 minutes adsorption, the inoculum was removed and the cells were washed twice with medium containing 3% FBS. At various times after infection, duplicate wells were harvested, the medium was adjusted to 100 mM MgSO₄ and 50 mM HEPES (pH 7.5), and the cells and medium were quickly frozen and thawed three times. The medium was clarified by centrifugation and the virus was titrated. In HEp-2 cells, HRSV produced approximately 10-fold more virus than rBRSV (Fig. 3, Panel A), whereas in MDBK cells the situation was reversed (Fig. 3, Panel B). In MDBK cells, HRSV reached a maximum titer after 2 days, whereas rBRSV continued to grow and reached a maximum after 4 to 5 days. These results are consistent with partial restriction of growth for HRSV in this bovine cell line. Interestingly, the chimeric rBRSV/A2 replicated better than rBRSV by approximately 2-fold in both the human and bovine cell lines, and in the bovine cells its growth curve resembled that of rBRSV rather than HRSV. These results indicate that the growth characteristics of the chimeric virus were not identical to either parent, consistent with its chimeric nature. The ability to replicate efficiently in this multi-cycle growth experiment indicates that the HRSV G and F proteins are functional in the rBRSV background. Most importantly, these results indicate that the chimeric virus is fully competent for growth in two cell lines, one of human origin and one of bovine.

Immunoelectron microscopy was employed to examine virions for the presence of G protein of the appropriate specificity. MDBK cells were infected with an MOI of 0.1 of rBRSV, rBRSV/A2, or HRSV. At 72 hours post infection, when CPE was pronounced, the material was fixed with buffered 0.05% glutaraldehyde for 15 min to stabilize the virion structure. The monolayer was scraped off the plate and after low speed centrifugation, the pellet was resuspended in a small amount of buffer. Glow discharged formvar coated 300 mesh copper grids, stabilized with carbon, were floated on drops of cell suspension. Nonspecific adsorption of antibodies was blocked by treating the grids with 1% cold water fish gelatin (Sigma, Deisenhofen, Germany) in standard phosphate buffered saline (PBS) containing 1% bovine serum albumin (PBS-BSA). Subsequently, the grids were floated for 45 min on drops of monoclonal antibodies (2F, specific to HRSV and

BRSV F, 44F, specific to HRSV F, 021/1G, specific to HRSV G, and 66G, specific to BRSV G (Garcia-Barreno et al., *J. Virol.* 63:925-932, 1989; Martinez et al., *J. Gen. Virol.* 79:2215-2220, 1998)), diluted in PBS-BSA. After several washings in PBS-BSA, bound antibodies were detected with colloidal gold (10 nm) labeled goat anti-mouse immunoglobulin Fab (GAF10, BioCell Int., Cardiff, United Kingdom) and negatively stained with phosphotungstic acid (PTA), pH 6.0. The grids were examined using a 400 T transmission electron microscope (Philips, The Netherlands). This process leaves virion structure intact and allows detection of external surface antigens.

Electron microscope analyses revealed large amounts of filamentous particles with a diameter of 100 to 200 nm and of several μ m in length in preparations of rBRSV (Fig. 4, Panel A), rBRSV/A2, and HRSV strain Long. Comparison of material after ultrathin section and after glutardialdehyde fixation proved that the shape of the virions was maintained after glutardialdehyde fixation. Variations in the virion diameter that are observed after glutardialdehyde fixation might be due to dehydration of the particles during the fixation procedure (Fig. 4, Panels A and B). Furthermore, the antigenic structure and reactivity of monoclonal antibodies was unaffected (Fig. 4, Panel A, insert).

The virus preparations were subjected to immunoelectron microscopy using the set of antibodies directed to BRSV and HRSV G and F proteins (Table 1), followed by labeling with gold-conjugated goat anti-mouse immunoglobulin, and negatively stained. Using a monoclonal antibody directed to HRSV F (Fig. 5, Panels B, D and F), a dense immunogold labeling of the rBRSV/A2 virion surface could be observed (Fig. 5, Panel D), comparable to the labeling intensity of the HRSV virion surface (Fig. 5, Panel F), whereas no specific labeling of rBRSV virions was detected (Fig. 5, Panel B). Antibody 2F, reacting with both BRSV and HRSV F protein (Fig. 5, Panels A, C and E), was used as control to demonstrate the intact antigenic structure of all virus preparations, including the BRSV preparation. This confirmed that the rBRSV/A2 chimeric virus and HRSV positive control virus contained the HRSV F protein, whereas the rBRSV negative control virus did not, as expected.

A monoclonal antibody directed to HRSV G protein reacted with rBRSV/A2 (Fig. 6, Panel D), as well as with HRSV particles (Fig. 6, Panel F), and failed to react with the rBRSV virion surface (Fig. 6, Panel B). When using an antibody directed to BRSV G as a control, only the rBRSV virion surface was immunogold labeled (Fig. 6, Panel A), but not

EXAMPLE III

Construction of cDNA encoding a chimeric BRSV/HRSV virus containing a single gene replacement involving a different HRSV subgroup A virus.

5 The chimeric rBRSV/A2 virus described above involved the simultaneous substitution of two surface glycoproteins, G and F. It was of interest to determine whether a BRSV/HRSV chimeric virus could be made in which the substitution involved only one of the two glycoproteins, and whether the substitution could be made with a different subgroup A strain, namely the Long strain. A second BRSV antigenome cDNA was constructed which
10 contains the HRSV strain Long F gene in place of the BRSV F gene (Fig.s 7A and 7B). Total RNA was made from HEp-2 cells infected with HRSV strain Long. Subsequently, RT-PCR was performed using primers FL_a
(5'-AGGAATTCGCATGCGGGGCAAATAACAATGGAGTTGCCAATC-3' (SEQ ID NO.: 21), nt 1 to 28 of the HRSV strain Long F gene sequence, containing an EcoRI/SphI
15 adapter, underlined) and FL_{Ra}
(5'-AGGAATTCTCGAGTTTTTATATAACTATAAACTAGGAATCTAC-3' (SEQ ID NO.: 22), HRSV strain Long F gene nt 1903 to 1874, EcoRI/*Xho*I adapter underlined), yielding a PCR product of 1,930 bp. The EcoRI adapters were used to clone the PCR product into pBluescript SK- (Stratagene, La Jolla, CA). The PCR product was sequenced,
20 and a 1,906 bp fragment was excised with SphI and *Xho*I and transferred into the SphI-*Xho*I window of pBRSV18, yielding pBRSV/LongF-12. This encodes a chimeric RSV antigenome of 15,114 nt.

Plasmid pBRSV/LongF-12 was transfected onto BSR T7/5 cells stably expressing T7 RNA polymerase, as described above. Five days after transfection, a
25 cytopathic effect of rounding cells typical for RSV could be observed in all transfected dishes. The recovery rates of rBRSV, rBRSV/A2 and rBRSV/LongF were comparably high, typically yielding about 100 foci per 32-mm dish. Virus stocks were produced by two passages of the supernatants from transfections on MDBK cells.

To test the identity of recombinant BRSV containing the HRSV strain Long F
30 gene in place of the BRSV F gene sequence, two RT-PCRs were done (Fig. 8). A first RT-PCR (Fig. 8, PCR1) was done using a primer pair hybridizing with sequences of the BRSV backbone, upstream and downstream of the F gene, yielding products of 2,187 nt for

EXAMPLE IV

Growth of the rBRSV/A2 Chimeric Virus in Seronegative Juvenile Chimpanzees.

Young chimpanzees which were determined to be seronegative for HRSV were inoculated by both the intranasal and intratracheal routes with a dose of 10^7 pfu per ml of rBRSV or rBRSV/A2 at each site (Table 2). Each virus was administered to two chimpanzees. Following inoculation of the virus, nasopharyngeal swab samples were taken daily on days 1-10 and 12, and tracheal lavage samples were taken on days 2, 5, 6, 8 and 12. Specimens were frozen and RSV titers were measured later by plaque assay on HEp-2 cells. The amount of rhinorrhea, a measure of upper respiratory tract illness, was estimated daily and assigned a score of 0-4 (0=none, 1= trace, 2= mild, 3= moderate, 4= severe). The results were compared to historic controls of animals which had received (i) 10^4 pfu of recombinant HRSV strain A2 wild type virus per site (Whitehead, et al., J. Virol. 72:4467-4471, 1998, incorporated herein by reference) or (ii) 10^5 pfu of the live-attenuated rA2cp28/404 strain A2 vaccine candidate per site (Whitehead, et al., J. Virol. 73:3438-3442, 1999, incorporated herein by reference), administered by the same routes (Table 2).

Table 2. Replication of recombinant rBRSV and chimeric rBRSV/A2 in the upper and lower respiratory tract of chimpanzees

Identification	Nasopharyngeal swab titer (log ₁₀ pfu/ml)													Tracheal lavage titer (log ₁₀ pfu/ml)						Peak rhinorrhea score
	No.	Day post-inoculation												Day post-inoculation						
Chimpanzee	Virus	1	2	3	4	5	6	7	8	9	10	12	Peak	2	5	6	8	12	Peak	
95A016	rBRSV ^a	<0.7	<0.7	<0.7	<0.7	<0.7	<0.7	<0.7	<0.7	<0.7	<0.7	<0.7	<0.7	<0.7	<0.7	<0.7	<0.7	<0.7	<0.7	0
95A018	rBRSV ^a	<0.7	<0.7	<0.7	<0.7	<0.7	<0.7	<0.7	<0.7	<0.7	<0.7	<0.7	<0.7	<0.7	<0.7	<0.7	<0.7	<0.7	<0.7	0
	mean												<0.7						<0.7	0.0
95A007	rBRSV/A2	<0.7	<0.7	0.7	0.7	1.0	<0.7	<0.7	<0.7	<0.7	<0.7	<0.7	1.0	<0.7	0.7	1.2	<0.7	<0.7	1.2	0
95A008	rBRSV/A2	<0.7	<0.7	0.7	<0.7	1.4	0.7	<0.7	<0.7	0.7	1.2	<0.7	1.4	<0.7	0.7	2.0	<0.7	<0.7	2.0	0
	mean												1.2						1.6	0.0
rA2cp248/404 ^b		<0.7	<0.7	1.1	2.2	1.5	2.0	1.4	1.4	1.0	0.7	<0.7	2.5 ^d	<0.7	1.0	1.0	<0.7	<0.7	1.4 ^d	0.8
	mean																			
rA2 ^c		<0.7	1.7	2.6	4.5	4.4	4.4	4.4	4.4	1.6	<0.7	<0.7	4.9 ^d	2.0	4.2	3.3	2.2	<0.7	4.7 ^d	2.5
	mean																			

^a Each virus was administered at a dose of 10⁷ pfu/ml in a 1.0 ml inoculum per site intranasally and intratracheally. Nasopharyngeal swab samples were collected on days 1-10 and 12 and tracheal lavage samples were collected on days 2, 5, 6, 8 and 12.

^b Virus rA2cp248/404 was evaluated in 4 chimpanzees at a dose of 10⁵ pfu/ml. (Whitehead, et al., *J. Virol.* 73:3438-3442, 1999).

^c Virus rA2 (rA2sites) was evaluated in 4 chimpanzees at a dose of 10⁴ pfu/ml. (Whitehead, et al., *J. Virol.* 72:4467-4471, 1998).

^d Mean peak titers are calculated using the peak virus titer achieved in each animal. Because peak virus titers were achieved on different days for each animal, the mean peak titer exceeds that of the mean titer on any given day.

Table 3. Replication of w/RSV A2 challenge virus in chimpanzees previously inoculated with either rBRSV or rBRSV/A2^a

Chimp	Virus	Nasopharyngeal swab titer (log ₁₀ pfu/ml)					Tracheal lavage titer (log ₁₀ pfu/ml)					Peak rhinorrhea score
Identifi- cation	used for immuniza- tion	Day post-inoculation					Day post-inoculation					Peak
No.		0	3	5	7	10	Peak	3	5	7	10	
95A016	rBRSV	<0.7	3.7	4.1	1.0	<0.7	4.1	5.2	6.0	<0.7	<0.7	6.0 1
95A018	rBRSV	<0.7	3.8	3.0	<0.7	<0.7	3.8	2.0	4.5	<0.7	<0.7	4.5 1
	mean						4.0					5.3 1.0
95A007	rBRSV/A2	<0.7	4.6	4.3	1.6	<0.7	4.6	5.7	6.3	1.7	<0.7	6.3 2
95A008	rBRSV/A2	<0.7	2.2	5.4	1.7	<0.7	5.4	3.2	6.3	2.8	<0.7	6.3 2
	mean						5.0					6.3 2.0

^a Each chimpanzee was challenged on day 30 with w/RSV A2 administered intranasally and intratracheally at a dose of 10⁴ pfu/ml in a 1.0 ml inoculum per site. Nasopharyngeal swab samples and tracheal lavage samples were collected on the indicated days.

EXAMPLE V

Construction of a Chimeric BRSV/HRSV Containing the HRSV G and F Genes in a Promoter-Proximal Shifted Position

The present example describes construction of an infectious rBRSV/HRSV chimera in which the HRSV G and F genes are substituted into a recombinant bovine RSV (rBRSV) background. The resulting human-bovine chimera contains two genes of HRSV, namely G and F, and eight genes from BRSV, namely NS1, NS2, N, P, M, SH, M2 and L. In addition to this basic substituted glycoprotein construction, the HRSV G and F genes were also shifted to a more promoter-proximal position in the rBRSV backbone, i.e., relative to a wild-type gene order position of the F and G genes in the RSV genome. More specifically, the F and G genes were moved from their usual location relative to the promoter, namely gene positions 7 and 8, respectively, to positions 1 and 2, respectively.

Complete infectious rBRSV was produced as above in which nucleotide substitutions were made to create unique *NotI*, *SalI* and *XhoI* sites at positions 67, 4,673 and 7,471, respectively (Fig 10A) (Buchholz et al., *J. Virol.* 73:251-259, 1999; Buchholz et al., *J. Virol.* 74:1187-1199, 2000, incorporated herein by reference). The *NotI* site is contained within the upstream nontranslated region of the BRSV NS1 gene, and the *SalI* and *XhoI* sites are in intergenic regions. Digestion of the rBRSV antigenomic cDNA with *SalI* and *XhoI* excised the BRSV G and F genes in their entirety and created compatible cohesive ends that were ligated. This resulted in an rBRSV antigenomic cDNA lacking the G and F genes and containing a 64-nucleotide SH-M2 intergenic region with the following sequence:

TTAAACTTAAAAATGGTTTATGtcgaGGAATAAAATCGATTAACAACCAATCAT
TCAAAAAGAT (SEQ ID NO. 23) (the tetranucleotide cohesive ends of the original cleaved *SalI* and *XhoI* sites are in small case). For comparison, the naturally-occurring BRSV F-M2 intergenic sequence is 55 nucleotides in length.

A cDNA containing the HRSV G and F genes was prepared by PCR with mutagenic primers used to modify the cDNA ends. Specifically, PCR was used to amplify nucleotides 4692-7551 of the complete HRSV antigenomic cDNA (spanning from the ATG of the G ORF to the end of the F gene-end signal), and the primers were

designed to add, immediately after the F gene-end signal, the first 6 nucleotides of the F-M2 IG followed by a copy of the NS1 gene-start signal. The PCR primers also were designed to add a *BspI* site and an *NotI* site each on both ends of the cDNA. The sequence of the cDNA fragment that was subjected to PCR was confirmed by
5 dideoxynucleotide sequencing. This cDNA was then inserted as a *NotI* fragment into the unique *NotI* site of the rBRSV antigenomic cDNA lacking the G and F genes as described above. A correct recombinant was identified by restriction fragment mapping, and was designated rBRSV/A2-G1 F2. The structure of the encoded genomic RNA is shown in Fig. 10 C. As shown, in this cDNA the G and F genes were moved from positions 7 and
10 8 relative to the promoter to positions 1 and 2.

A plasmid encoding the antigenomic RNA of rBRSV/A2-G1 F2 was transfected, together with plasmids encoding the N, P, M2-1 and L support proteins, into BSR T7/5 cells, which stably express the T7 RNA polymerase, as described above (see also, Buchholz et al., *J. Virol.* 73:251-259, 1999; Buchholz et al., *J. Virol.* 74:1187-1199,
15 2000, each incorporated herein by reference), and infectious virus was recovered. The recovered rBRSV/A2-G1F2 virus was compared to rBRSV, rHRSV (also called rA2) and rBRSV/A2 with regard to the efficiency of multicycle growth in human HEp-2 cells and bovine MDBK cells. As described above (see also, Buchholz et al., *J. Virol.* 74:1187-1199,
20 2000, incorporated herein by reference), rHRSV grows much more efficiently than rBRSV in HEp-2 cells, and the rBRSV/A2 virus grows with an efficiency intermediate between that of each parent. As shown in Fig. 11, the efficiency of replication of rBRSV/A2-G1F2 was indistinguishable from that of rBRSV/A2. Thus, unexpectedly, the change in the location of the G and F genes did not reduce the efficiency of growth *in vitro*, which novel result allows for efficient production of RSV vaccine virus.

Immunofluorescence was performed on HEp-2 cells infected with wt HRSV or the chimeric viruses rBRSV/A2 or rBRSV/A2-G1F2. This was performed using two monoclonal antibodies specific to the HRSV G or F proteins, namely 021/01G and 44F, respectively (Lopez et al., *J. Virol.* 72:6922-6928, 1998; Melero et al., *J. Gen. Virol.* 78:2411-2418, 1997, each incorporated herein by reference). The staining was
30 done with each monoclonal antibody individually. Although this assay is only semi-quantitative, it has been previously determined that the assay distinguishes reliably between wt rBRSV and rBRSV/A2 (the latter bearing the HRSV G and F genes in the

normal genome location in the rBRSV backbone). In particular, wt HRSV gives a very strong, extensive pattern of immunofluorescence indicative of efficient and extensive antigen expression, while rBRSV/A2 gives a weaker, more diffuse, less extensive pattern (Buchholz et al., *J. Virol.* 74:1187-1199, 2000, incorporated herein by reference). A comparable assay conducted for rBRSV/A2-G1F2 (Fig. 12), shows that the pattern of immunofluorescence for this promoter-shifted chimeric virus was very similar to that of wt HRSV. This result is consistent with increased expression of the G and F glycoproteins. At the same time, the cytopathic effect associated with rBRSV/A2-G1F2 was reduced compared to wt HRSV, and more closely resembled that of rBRSV/A2 as described previously (Buchholz et al., *J. Virol.* 74:1187-1199, 2000, incorporated herein by reference). Specifically, rBRSV/A2 and rBRSV/A2-G1F2 induced fewer and smaller syncytia.

Thus, the present example documents modification of the rBRSV/A2 virus, which contains the genes for the major protective antigens of HRSV, the G and F proteins, in the background of BRSV which is strongly attenuated for replication in the respiratory tract of primates. As shown in the preceding example, the rBRSV/A2 virus has a strong host range restriction that renders it highly attenuated in primates. Since the present rBRSV/A2-G1F2 virus bears the same constellation of BRSV genes in its genetic background, it is likely to share this strong host range restriction phenotype, thereby increasing the expression of the two major protective antigens. The increased expression of these two protective antigens in vivo is further expected to increase the immunogenicity of this virus. Thus, the present example modified and improved the rBRSV/A2 virus by moving the HRSV genes to a promoter proximal location. A positional shift of this magnitude, i.e., where the G and F genes were moved from wild type positions 7 and 8 relative to the promoter to new positions 1 and 2, has not been described previously.

EXAMPLE VI

Construction of a Chimeric BRSV/HRSV With Envelope-Associated M, G and F Proteins Derived From HRSV

The present example demonstrates yet another human-bovine chimeric

As indicated by the examples herein, genes to be replaced in human-bovine chimeric RSV can be selected that are likely to interact functionally or structurally based on available knowledge of RSV structure/function. These exchanges and other modifications within human-bovine chimeric RSV are further simplified by the fact that proteins that interact are juxtaposed in the genome, for example the N and P nucleocapsid proteins, the M, SH, F and G envelope proteins, and the M2-1, M2-2 and L polymerase components. Thus, additional candidate vaccine strains according to the invention can be achieved, for example, by incorporating two or more juxtaposed genes, e.g., selected from N and P, two or more of the M, SH, F and G envelope genes, or two or more of the M2-1, M2-2 and L genes, together as a heterologous insert or substitution unit in a recipient or background genome or antigenome.

For example, the M and SH genes can be replaced together in rBRSV/A2 with their HRSV counterparts. This will result in a virus in which the viral envelope proteins (G, F, SH and M) are all of HRSV, while the internal proteins are of BRSV. This can be followed, as needed, by replacement of additional BRSV genes with their human counterparts, for example, N and P as another pair, NS1 and NS2 as another, and M2-1, M2-2 and L as another group. The juxtaposition of each pair of genes will simplify the substitutions. At the same time, the converse approach of inserting individual BRSV genes into HRSV, leaving the HRSV G and F antigenic determinants undisturbed, will also yield desired vaccine candidates within the invention. For example, one or more of the N, P, M2-1 and M genes of a human RSV can be individually replaced by their bovine counterparts. Recovered recombinant viruses are then evaluated for the attenuation phenotype in cell culture, rodents, and nonhuman primates, as exemplified herein. In this manner, the invention provides for identification of candidate human-bovine chimeric RSV vaccine viruses having desired levels of attenuation and protective efficacy for treatment and prophylaxis of RSV in various subjects.

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